Title	Hypolipidemic effects of wx/ae double-mutant rice and its components in model mice
Author(s)	Nakaya, Makoto
Editor(s)	
Citation	
Issue Date	2014
URL	http://hdl.handle.net/10466/14381
Rights	

Hypolipidemic effects of *wx/ae* double-mutant rice and its components in model mice

2014

Makoto Nakaya

TABLE OF CONTENTS

CHAPTER II

Hypolipidemic Effects of Starch and γ-Oryzanol from *wx/ae* Double-mutant Rice in BALB/c.KOR-*Apoe*^{shl} Mice

ABBREVIATIONS

AACC	American Association for Clinical Chemistry
ae	*amylose-extender*
ALT	alanine aminotransferase
AMG	amyloglucosidase
ANOVA	analysis of variance
apoE	apolipoprotein E
AST	aspartic aminotransferase
BALB/c.KOR-*Apoe*^{shl}	BALB/c.KOR/Stm Slc-*Apoe*^{shl}
°C	degree Celsius
C group	control group
CAD	charged aerosol detector
CYP2R1	cytochrome P450, family 2, subfamily R, polypeptide 1
DS	digestible starch
EF-1α	elongation factor-1α
FAME	fatty acid methyl esters
FAS	fatty acid synthetase
GBSS I	granule-bound starch synthase I
GLP-1	glucagon-like peptide-1
HDL	high-density lipoprotein
HMG-CoAR	3-hydroxy-3-methylglutaryl coenzyme A reductase
HPLC	high performance liquid chromatography
I.D.	inside diameter
ICR	Institute of Cancer Research

LDL	low-density lipoprotein
LDL-R	low-density lipoprotein receptor
LPL	lipoprotein lipase
LXRs	liver X receptors
NEFA	non-esterified fatty acids
NFE	nitrogen free extract
NSY	Nagoya-Shibata-Yasuda
O group	γ-oryzanol group
PCR	polymerase chain reaction
PPAR-α	peroxisome proliferator-activated receptor-α
PYY	peptide YY
RS	resistant starch
RSO group	resistant starch plus γ-oryzanol group
SBE IIb	starch branching enzyme IIb
SCFA	short-chain fatty acids
SD	standard deviation
shl	spontaneously hyperlipidemic
SHP	small heterodimer partner
SREBP	sterol regulatory element-binding protein
TAG	triacylglycerol
TBARS	thiobarbituric acid reactive substances
U	unit
UV	ultraviolet
VWD	variable wavelength detector
WT	wild-type
wx	*waxy*
wx/ae	*waxy/amylose-extender*

GENERAL INTRODUCTION

In recent years, the number of patients with lifestyle-related diseases such as type 2 diabetes, stroke, heart disease, hyperlipidemia, hypertension, atherosclerosis, and obesity has increased all over the world [1, 2]. These diseases all involve abnormal glycometabolism and lipid metabolism [3, 4]. Many supplements and functional foods are available to treat these diseases [5, 6], but I speculate that improving lipid metabolism is the most effective strategy.

In Japan, rice is traditionally the principal food and we have good cultivation techniques for rice plants, so it would be good if we can make supplements or functional foods from rice. *Japonica* rice (*Oryza sativa* L.) *waxy/amylose-extender* (*wx/ae*) double-mutant line AMF18 is derived from a cross between a *waxy* (*wx*) mutant line EM21 and an *amylose-extender* (*ae*) mutant line EM16, and is defective in *starch branching enzyme IIb* (*SBE IIb*) and *granule-bound starch synthase I* (*GBSS I*) genes, resulting in its producing a unique starch of pure amylopectin with branched chains longer than those of the parental rice cv. Kinmaze (wild-type, WT) [7-9]. Kubo *et al.* give the structure, physical and digestive properties of *wx/ae* starch [7, 8], X-ray diffraction analysis revealed that the crystalline structure of *wx/ae* starch is B type, unlike WT starch which is A type. *wx/ae* starch starts gelatinization at a higher temperature than WT starch and retrogrades quickly. The digestion rate of raw *wx/ae* starch using porcine pancreatic α-amylase *in vitro* is

markedly slower than that of WT starch, but the digestion rates of a gelatinized *wx/ae* starch and gelatinized WT starch are the same. To determine *in vivo* digestibility, we measured the postprandial blood glucose response of rats to ingestion of raw *wx/ae* starch. *wx/ae* starch suppressed an increase of postprandial blood glucose concentration ordinarily observed after eating WT starch. These results suggest that *wx/ae* starch has a high amount of resistant starch due to the elongated chains in *wx/ae* amylopectin. Table 1 shows the nutritional constituents of WT and *wx/ae* brown rice. *wx/ae* brown rice has higher sucrose, lipid and dietary fiber content than WT brown rice. Rice lipids contain lipid-soluble functional materials such as γ-oryzanol. γ-Oryzanol is known to have beneficial effects on lipid metabolism [10, 11].

wx/ae rice is expected to contain highly beneficial nutrients for glycometabolism and lipid metabolism, including resistant starch and γ-oryzanol. The objective of the present study is to examine the effect of *wx/ae* double-mutant rice and its components on lipid metabolism in two types of lifestyle-related disease model mice.

Table 1. Nutritional constituents of WT and *wx/ae* brown rice

Constituent	WT brown rice	*wx/ae* brown rice
NFE * (%)	69.8	58.9
Sucrose (%)	0.78	3.20
Protein (%)	7.3	7.8
Lipid (%)	3.1	5.0
Dietary fiber (%)	2.8	8.2
Ash (%)	1.4	1.7
Moisture (%)	15.6	18.4
Energy (kcal/100 g)	342	328

Rice plants were grown in the summer of 2007 in an experimental field at Osaka Prefecture University. The nutritional composition of each brown rice was analyzed by Japan Food Research Laboratories (Suita, Osaka). *NFE: nitrogen free extract (carbohydrate and others)

CHAPTER I

wx/ae Double-Mutant Brown Rice Prevents the Rise in Plasma Lipid and Glucose Levels in Mice

1-1 Introduction

Dyslipidemia and hyperglycemia are characteristic of metabolic syndrome, which appears to promote development of atherosclerotic cardiovascular disorder as well as increasing the risk of developing type 2 diabetes [12]. Food materials that are multi-effective against symptoms of metabolic syndrome are needed to prevent and treat metabolic syndrome, and research and development of such foods are in progress throughout the world [13, 14].

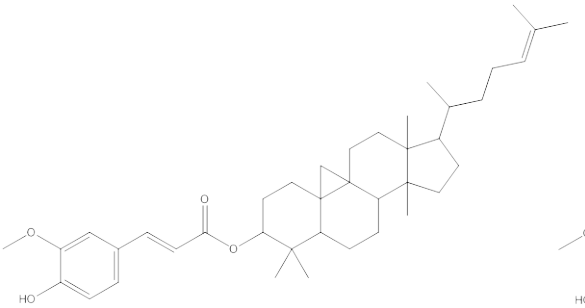
A *wx/ae* double mutant AMF18 is generated by crossing amylose-free *waxy* (*wx*) mutant EM21 and *amylose-extender* (*ae*) mutant EM16 cultivars [7]. *wx/ae* rice starch lacks amylose and is composed of long-unit chains of amylopectin, making it hard to digest *in vitro* and *in vivo* [8]. Adding resistant starch (RS) to the diet improves insulin sensitivity in people with either normal or impaired glucose tolerance [15, 16]. RS stimulates the endogenous secretion of glucagon-like peptide-1 (GLP-1) and peptide YY (PYY), a possible mechanism for improving insulin sensitivity by RS [17]. Lattimer and Haub have reported that the intake of rice bran reduced plasma cholesterol levels and

improved plasma cholesterol status [18], suggesting that *wx/ae* brown rice may also be useful in preventing both type 2 diabetes and hypercholesterolemia.

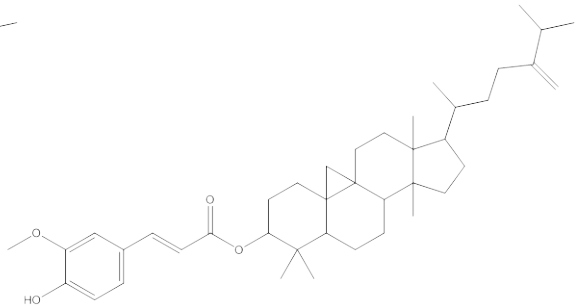
γ-Oryzanol is one of the major functional components in rice bran [19, 20]. γ-Oryzanol comprises a mixture of phytosteryl ferulates as shown Figure 1-1 and has been shown to have beneficial effects on hypercholesterolemia, hyperlipidemia, and insulin resistance in animal models [21, 22]. It also has antiinflammatory and antioxidant effects [23]. Rice cultivars with a high γ-oryzanol content might therefore be valuable for preventing and treating various diseases.

Nagoya-Shibata-Yasuda (NSY) mice have been established as an inbred animal model of spontaneous type 2 diabetes by selective breeding for glucose intolerance from outbred ICR mice[24]. NSY mice have been used in pharmaceutical and food research [14, 25], since the clinical characteristics of NSY mice resemble those of the common forms of type 2 diabetes and insulin resistance [26, 27].

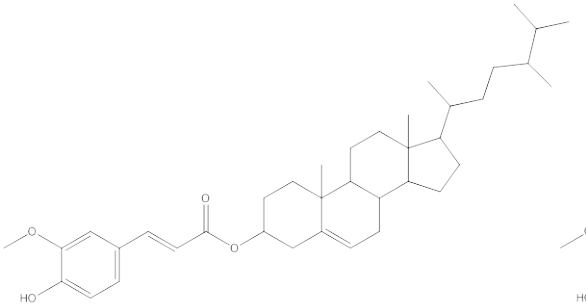
In chapter I, I compared the effects of *wx/ae* brown rice on lipid and glucose metabolism to those of Koshihikari brown rice, one of the most popular non-waxy *japonica* rice cultivars, by using high-fat diet-fed type 2 diabetic NSY/Hos mice.



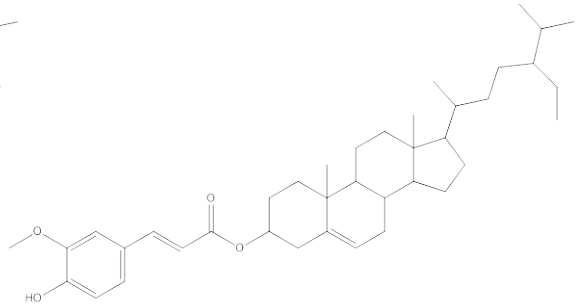
cycloartenyl ferulate



24-methylenecycloartenyl ferulate



campesteryl ferulate



β-sitosteryl ferulate

.

1-2 Materials and Methods

1-2-1 Preparation of the brown rice powder

wx/ae and Koshihikari rice plants were respectively grown in the summer of 2010 in Yamanashi and Ishikawa prefectures of Japan. Both types of brown rice were washed with water at room temperature and dried at 70 °C for 2 h with a DSJ-7 electric drying machine (Shizuoka-seiki, Fukuroi, Shizuoka, Japan). Each type of rice was powdered with a WB-1 Wonder blender (Osaka Chemical, Osaka, Japan) and then used for animal experiments. The nutritional composition of each type of brown

rice was analyzed by Sunatec (Yokkaichi, Mie, Japan) as shown in Table 1-1. Dietary fiber was measured by using a total dietary fiber assay kit (Megazyme, Wicklow, Ireland) based on AACC method 32-05.

Table 1-1. Nutritional constituents of each brown rice

Constituen	Koshihikari brown rice	*wx/ae* brown rice
Protein (%)	6.7	7.4
Lipid (%)	3.5	5.3
NFE* (%)	74.1	67.7
Digestible starch (%)	66.9	26.5
Resistant starch (%)	< 2.0	27.8
Dietary fiber (%)	4.9	9.0
Ash (%)	1.3	1.6
Moisture (%)	9.5	9.0
γ-Oryzanol (μg/g)	268	514
Energy (kcal/100g)	364.5	366.1

*NFE: nitrogen free extract (carbohydrate and others)

1-2-2 Measurement of resistant starch and digestible starch

RS and digestible starch (DS) were measured using an RS assay kit (K-RSTAR, Megazyme; AOAC Method 2002.02, AACC Method 32-40). This method can give precise results for over 2%

RS content. Four milliliters of pancreatic α-amylase (10 mg/ml) containing amyloglucosidase (AMG, 3 U/ml) was added to 100 mg brown rice powder samples, and then the samples were incubated at 37 °C while continuously shaking for 16 h. After adding ethanol, each sample was centrifuged to separate DS and RS. The resulting supernatant was collected as a DS solution. A 2-mL amount of 2 M KOH was subsequently added to the precipitate, and the dissolved sample was hydrolyzed at 50 °C for 30 min by adding an AMG solution (3300 U/ml). After centrifugation, the supernatant was collected as an RS solution. The glucose concentrations in the DS and RS solutions were determined by the glucose oxidase-peroxidase method and converted to starch contents by multiplying by 0.9. Each sample was analyzed three times.

1-2-3 Determination of the γ-oryzanol content

One gram of each type of brown rice powder was soaked in 10 g of a soaking solution (methanol/acetonitrile/acetic acid, 52/45/3 (v/v/v)) while gently stirring for 2 h. The extracted γ-oryzanol solution was collected on a no. 2 filter paper (Advantec, Tokyo, Japan). The γ-oryzanol content of each extracted solution was determined by high-performance liquid chromatography (HPLC). The reverse-phase HPLC system consisted of a PU-2089 pump (Jasco, Tokyo, Japan) equipped with a Cadenza CD-C18 column (250 mm x 4.6 mm I.D.; Imtakt, Kyoto, Japan), a CO-8010 column oven (Tosoh, Tokyo Japan), and an SPD-10A UV-VIS detector (Shimadzu, Kyoto, Japan). The conditions used were as follows: flow rate, 0.8 mL/min; oven

temperature, 30 °C; injection volume, 5 μL; detection, UV at 320 nm; and mobile phase, methanol/acetonitrile/acetic acid (52/45/3 v/v/v) [28, 29]. The data were produced in triplicate and with reference to the peak area-concentration calibration curve from authentic γ-oryzanol standards (Tokyo Chemical Industry, Tokyo, Japan). I confirmed by the same method that each extracted residue did not contain γ-oryzanol.

1-2-4 Diet and animals

Six-week-old male type 2 diabetic mice (NSY/Hos strain) were purchased from Hoshino Laboratory Animals (Bando, Ibaraki, Japan). After 2 weeks on a AIN-76-modified high-fat diet (milk casein, 28.2%; corn oil, 6.0%; beef tallow, 10.0%; sucrose, 22.0%; cellulose, 3.8%; DL-methionine, 0.3%; choline bitartrate, 0.2%; AIN-76 vitamin mix, 1.0%; AIN-76 mineral mix, 3.5%; α-cornstarch, 25.0%; energy 438.1 kcal/100g), the mice were assigned to two groups of six each based on body weight and fasting blood glucose level. The mice were individually housed and fed for 10 weeks with the AIN-76-modified high-fat diet containing each type of brown rice powder (25.0% w/w) instead of α-cornstarch. Their body weight and food intake were measured every week. The fasting blood glucose level was measured biweekly from the tail vein after 8 h of fasting by the glucose oxidase method, using a G-checker (Gunze, Kyoto, Japan). The mice were fasted for 14 hr before euthanasia was applied with CO_2 gas. Blood samples and organ samples were harvested and subsequently dissected for analysis. All experiments were conducted in accordance with the guidelines

for the proper conduct of animal experiments by the Science Council of Japan (2006).

1-2-5 Blood chemistry

Plasma total cholesterol, high-density lipoprotein (HDL) cholesterol, triacylglycerol (TAG), non-esterified fatty acids (NEFA), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were respectively analyzed by cholesterol E, HDL cholesterol E, Triglyceride E, NEFA-C, and Transaminase CII kits (Wako Pure Chemical Industries, Osaka, Japan). Plasma non-HDL cholesterol, including chylomicron, very-low-density lipoprotein, and low-density lipoprotein cholesterols, was calculated from the total cholesterol and HDL cholesterol levels. Plasma insulin was measured by using an insulin assay kit (Shibayagi, Shibukawa, Gunma, Japan), and plasma adiponectin was measured by using an Adiponectin DuoSet kit (R&D Systems, Minneapolis, MN, USA). Plasma lipid peroxidation was analyzed by measuring thiobarbituric acid reactive substances (TBARS) with a TBARS assay kit (Cayman Chemical, Ann Arbor, MI, USA).

1-2-6 Hepatic cholesterol and triacylglycerol

Total lipids were extracted from 30-40 mg of liver tissue by the method described by Bligh and Dyer [30], and then dissolved in isopropanol. Hepatic total cholesterol and TAG were respectively analyzed by using cholesterol E and triglyceride E kits.

1-2-7 Measurement of glycosuria

Fresh urine samples were collected at 10 weeks, and the glycosuria level was analyzed by the glucose oxidase method, using a urine glucose test strip (Terumo, Tokyo, Japan). The pathological scores for glycosuria are defined as follows: 0, < 50 mg/dL; 1, 50-100 mg/dL; 2, 100-500 mg/dL.

1-2-8 Analysis of feces

Feces were collected for 48 h at 10 weeks. The collected feces were lyophilized, homogenized, and then re-lyophilized. Fecal cholesterol and bile acid were extracted at 65 °C for 4 h with 90% ethanol. Fecal TAG was extracted at 60 °C for 90 min with a buffer containing 10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% (v/v) TritonX-100, and 80% isopropanol. The cholesterol, bile acid, and TAG concentrations were respectively analyzed by using Cholesterol E, total bile acid (Wako Pure Chemical Industries), and triglyceride E kits.

1-2-9 Quantitative real-time RT-PCR

Total RNA samples of the liver tissues were isolated by using a QuickPrep total RNA extraction kit (GE Healthcare, Piscataway, NJ, USA). cDNA was prepared from 2 μg of each total RNA sample which was reverse-transcribed by using Super Script III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and oligo (dT) primers (Invitrogen). Each prepared cDNA sample was purified by using a PCR purification kit (Qiagen, Cambridge, MA, USA). Quantitative real-time RT-PCR was performed by a StepOne real-time PCR system (Life Technologies Japan, Tokyo,

Japan) using a Fast SYBR Green Master Mix (Life Technologies Japan) according to the manufacturer's instructions. The housekeeping transcript, elongation factor-1α (EF-1α), was used as an internal control because of its stable expression *in vivo* [31]. The primers used for each gene are listed in Table 1-2. The PCR products were evaluated by an analysis of their melting curves (data not shown), all experiments being performed in duplicate.

Table 1-2. Primers used in real-time RT-PCR

Gene	Forward(5'-3')	Reverse(5'-3')
EF-1α	AGACCAGCAAATACTATGTGACCA	TTCTTGGAGATACCAGCTTCAAAT
SREBP-1	TGCTCCTGTGTGATCTACTTCTTG	TGTAGGAATACCCTCCTCATAGCA
SREBP-2	GTACTGTCACTGGAGTCAGGTGCT	CTTCACAAAGACGCTCAAGACAAT
LXRs	CCTTCCTCAAGGACTTCAGTTACA	CATATGTGTGTTGCAGCCTCTCTA
PPAR-α	TGAAAGCAGAAATTCTTACCTGTG	TGTTCATGTTGAAGTTCTTCAGGT
SHP	ACATCACTGAACTCCTTGAAGACA	GTGTACACTGTATCCAAAGCAAGG
HMG-CoAR	CAAAGAACTGACAGGCTTAAATGA	TCCACGAGCTATATTTTCCCTTAC
CYP7A1	AGGTCTCTGAACTGATCCGTCTAC	CATTTGAAATAAGCTCCAGAAGGT
CYP7B1	CTGGAAAGCACTATTCTTGAGGTT	AAAGAGGGCTACAAAATCTCCTTT
CYP39A1	GCACTGCAGGTAAAGACAAGATTA	TGATTCAGAATTTTAACTGGCTTC
FAS	GCACAGCTCTGCACTGTCTACTAC	ATCCCAGAGGAAGTCAGATGATAG
LDL-R	ATCTCTCAGCTCCCAGTACTCCTA	AGAAGATGGACAGGAACCTCATAC
CYP2R1	ATTCCAGATATGGTAGAGGATGGA	TTCATAAGTGAATCGTTCTCCAAA

1-2-10 Statistics

Each result is expressed as the mean ± standard deviation. Independent samples *t*-tests or two-way repeated-measure ANOVA with a *post hoc* analysis by Dunnett's test were carried out by using Ekuseru-Toukei software version 2010 (SSRI, Tokyo, Japan). Differences were considered significant at $P < 0.05$.

1-3 Results

1-3-1 Prevention of the rise in plasma lipid level by *wx/ae* brown rice

Table 1-1 shows that *wx/ae* brown rice contained 27.8% RS, 9.0% dietary fiber, and 514 μg/g γ-oryzanol, while Koshihikari brown rice contained < 2.0% RS, 4.9% dietary fiber and 268 μg/g γ-oryzanol. These nutritional constituents are known to have beneficial effects on lipid and glucose metabolism [15-18, 21, 22], suggesting that an intake of *wx/ae* brown rice would prevent the development of dyslipidemia and hyperglycemia more effectively than an intake of Koshihikari brown rice. I demonstrated this hypothesis by examining the effect of *wx/ae* brown rice on lipid and glucose metabolism by using a high-fat diet-fed type 2 diabetic mouse model which developed both dyslipidemia and hyperglycemia [14, 25]. I fed AIN-76-modified high-fat diets containing 25% Koshihikari brown rice or *wx/ae* brown rice to type 2 diabetic NSY/Hos mice for 10 weeks. All

animals were in good health throughout the experimental period, and no such side effect as diarrhea was apparent (data not shown).

There were no differences between the two groups in daily intake, total energy intake, body weight gain, or organ weight (Table 1-3). Figure 1- tracks the body weight of the mice during the 10-week study period. The body weight of the *wx/ae* group was slightly lower than that of the Koshihikari group, but there was no statistically significant difference between the two groups ($P = 0.11$). In addition, no difference in the weight of the epididymal white adipose tissue between the two groups was apparent (Table 1-3), suggesting that *wx/ae* brown rice had no anti-obese effect. On the other hand, the plasma total cholesterol ($P < 0.001$), non-HDL cholesterol ($P < 0.01$), TAG ($P < 0.05$), and NEFA ($P < 0.001$) levels were significantly lower in the *wx/ae* group. The total cholesterol/HDL cholesterol ratio, a predictor of cardiovascular disease [32], was also lower in the *wx/ae* group than in the Koshihikari group ($P < 0.05$).

No significant differences were apparent in plasma HDL cholesterol, insulin, adiponectin, TBARS, or hepatic lipid levels, while AST ($P < 0.05$) and ALT ($P < 0.001$), markers for liver condition, were lower in the *wx/ae* group than in the Koshihikari group, suggesting that *wx/ae* brown rice might have exerted a hepatoprotective effect against high-fat diet-induced liver damage.

Table 1-3. Physiological parameters and blood chemistry

	Koshihikari	*wx/ae*
Number	6	6
Intake (g/d)	4.1 ± 0.2	4.1 ± 0.2
Total energy intake (kcal)	1246.8 ± 59.2	1249.7 ± 62.1
Initial body weight (g)	39.7 ± 3.0	38.6 ± 1.7
Final body weight (g)	55.0 ± 2.7	52.1 ± 3.1
Body weight gain (g)	15.3 ± 1.7	13.5 ± 2.7
Liver weight (g)	1.8 ± 0.1	1.7 ± 0.2
Kidney weight (g)	0.7 ± 0.0	0.7 ± 0.0
EWAT (g)	1.6 ± 0.3	1.9 ± 0.3
Hepatic lipid content		
TAG (mg/g)	46.4 ± 19.1	35.1 ± 10.3
Total cholesterol (mg/g)	4.5 ± 2.1	3.0 ± 1.1
Dried feces (mg/d)	368.9 ± 80.7	540.4 ± 54.7 [b]
Fecal lipid content		
Total cholesterol (mg/d)	4.4 ± 0.7	4.5 ± 1.2
Bile acid (μmol/d)	2.9 ± 0.9	2.8 ± 0.5
TAG (mg/d)	1.1 ± 0.6	5.8 ± 1.3 [c]
Blood chemistry		
Total cholesterol (mg/dL)	177.5 ± 13.0	133.9 ± 12.3 [c]
non-HDL cholesterol (mg/dL)	68.8 ± 16.2	35.6 ± 12.1 [b]
HDL cholesterol (mg/dL)	108.8 ± 14.4	98.3 ± 11.6
Total cholesterol/HDL cholesterol ratio	1.7 ± 0.2	1.4 ± 0.1 [a]
TAG (mg/dL)	254.4 ± 64.2	171.7 ± 34.6 [a]
NEFA (mEq/L)	1.5 ± 0.1	0.8 ± 0.2 [c]
Insulin (ng/mL)	6.4 ± 2.7	10.0 ± 5.7
Adiponectin (μg/mL)	5.2 ± 1.1	5.2 ± 0.6
TBARS (nmol/mL)	34.7 ± 7.0	35.5 ± 4.5
AST (Karmen unit)	154.3 ± 33.8	105.1 ± 18.6 [a]
ALT (Karmen unit)	45.4 ± 8.1	24.4 ± 5.6 [c]

[a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$ *vs*. Koshihikari group (independent samples *t*-test).

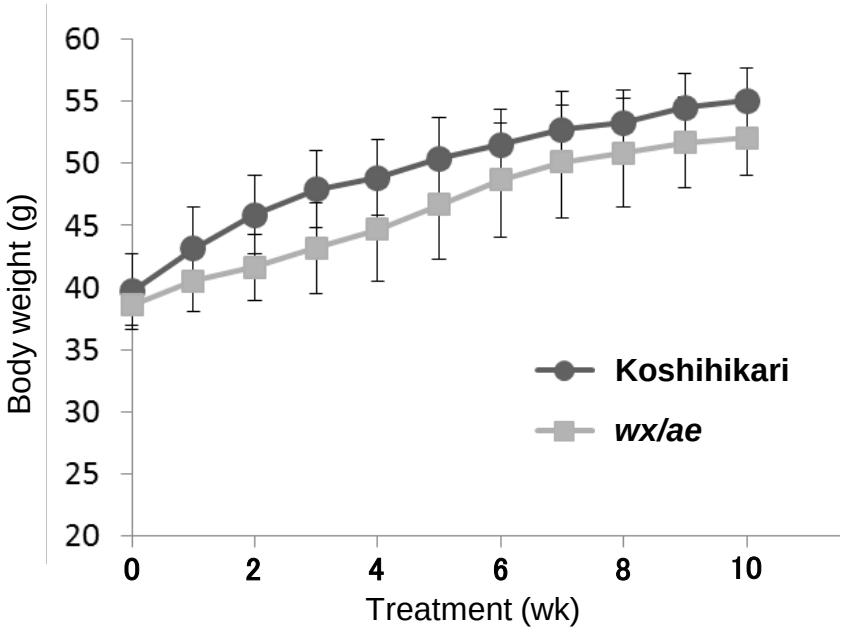


Figure 1-2. Mouse body weight during the 10-week study period.

Each value is presented as the mean ± standard deviation (n = 6). No statistically significant differences was apparent between the two groups (P = 0.1144, two-way repeated-measures ANOVA with a *post hoc* analysis by Dunnett's test).

1-3-2 Effect on fecal lipid excretion

Inhibiting dietary lipid absorption can induce favorable change in the plasma lipid content [33]. I analyzed the fecal lipid content to examine the effect of *wx/ae* brown rice on dietary lipid absorption (Table 1-3). The *wx/ae* group mice excreted more feces (P < 0.05), although the daily fecal excretion of cholesterol and bile acid were almost equal in both groups. Interestingly, the fecal TAG level in the *wx/ae* group was five times higher than that in the Koshihikari group (P < 0.001), indicating that dietary TAG absorption had been inhibited in the *wx/ae* group.

1-3-3 Effect on glycemic status

An intake of RS helps to prevent diabetes and insulin resistance [15, 16]. I analyzed the fasting blood glucose level throughout the experimental period and the glycosuria level at week 10 to identify the effects of *wx/ae* brown rice on glycemic status (Figure 1-3). The fasting blood glucose level was significantly lower in the *wx/ae* group than in the Koshihikari group ($P < 0.005$; Figure 1-3A). On the other hand, the pathological score for glycosuria at 10 weeks was significantly better in the *wx/ae* group than in the Koshihikari group ($P < 0.05$; Figure 1-3B). Accordingly, the glycemic status of the *wx/ae* group was judged superior to that of the Koshihikari group.

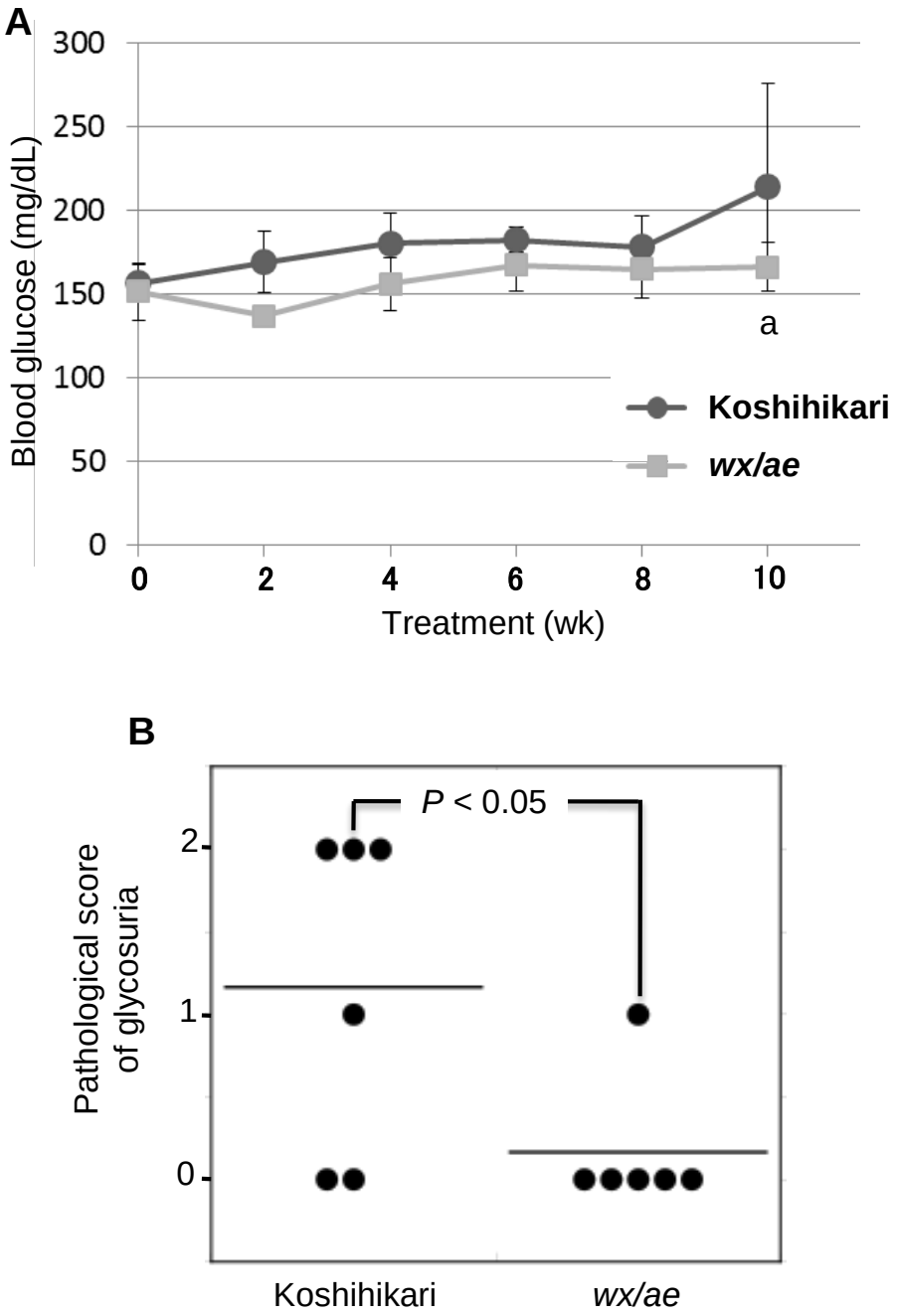


Figure 1-3. Effect on glycemic status.

A, Fasting blood glucose levels during the 10-week study period. Each value represented as the mean ± standard deviation (n = 6). Statistically significant difference between the two groups was apparent ($^{a}P < 0.005$, two-way repeated-measures ANOVA with a *post hoc* analysis by Dunnett's test). B, Pathological score for glycosuria at 10 weeks. The pathological data for glycosuria are scored as follows: 0, < 50 mg/dL; 1, 50-100 mg/dL; 2, 100-500 mg/dL. Means are shown as bars, and statistical results are shown in the graph (independent samples *t*-test).

1-3-4 Gene expression analysis in the liver

Treatment with hypolipidemic agents can influence the expression of hepatic genes related to lipid metabolism [14, 25]. I performed a SYBR green-based real-time RT-PCR analysis of the liver to compare the expression levels of lipid metabolism-related genes between the two groups (Figure 1-4). Among the 12 genes examined, the sterol regulatory element-binding protein (SREBP)-1, SREBP-2, fatty acid synthetase (FAS), low-density lipoprotein receptor (LDL-R), and cytochrome P450, family 2, subfamily R, polypeptide 1 (CYP2R1) genes were significantly upregulated in the *wx/ae* group. 3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoAR), the rate-limiting enzyme for cholesterol synthesis, tended to be higher in the *wx/ae* group ($P = 0.09$). In contrast, the gene expression levels of the liver X receptors (LXRs), regulators of lipid and glucose homeostasis [34], peroxisome proliferator-activated receptor-α (PPAR-α), a regulator of lipid oxidization, and enzymes related to bile acid synthesis, including small heterodimer partner (SHP), CYP7A1, CYP7B1 and CYP39A1 [34, 35], were unchanged in the *wx/ae* group. These results indicate that lipogenesis, but not bile acid synthesis, was upregulated in the *wx/ae* group.

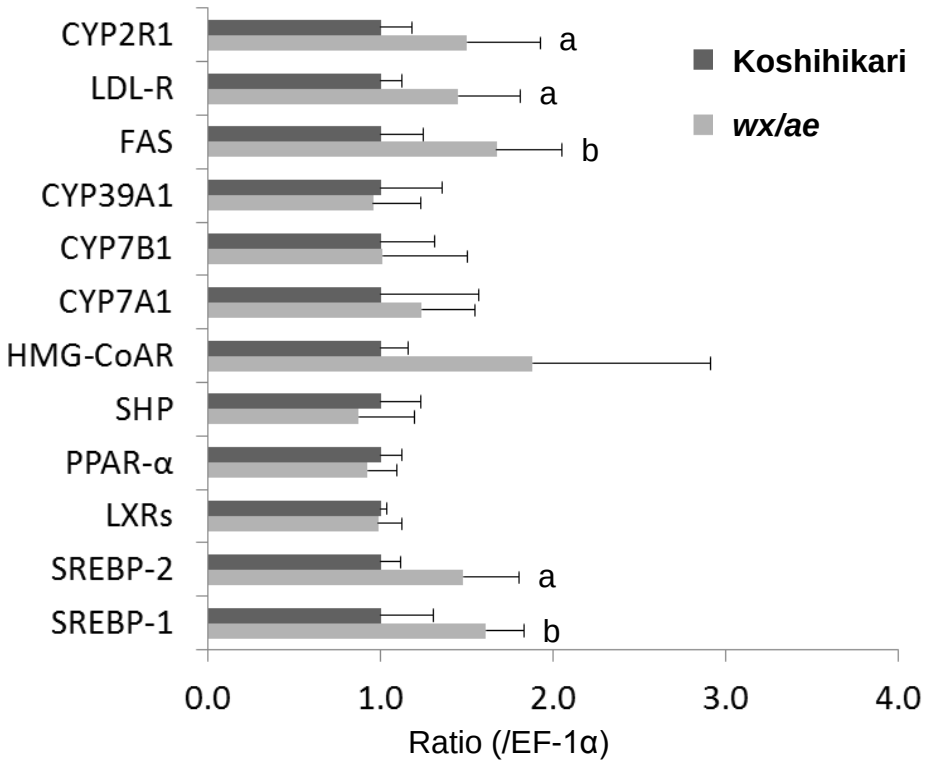


Figure 1-4. Gene expression analysis of the liver by SYBR green-based real-time RT-PCR.

Elongation factor-1 alpha (EF-1α) was used as a control to standardize the efficiency of each reaction. Each value is presented as the mean ± standard deviation (n = 6; $^{a}P < 0.05$, $^{b}P < 0.01$, independent samples t-test).

1-4 Discussion

A *wx/ae* double-mutant rice generated by crossing *wx* mutant line EM21 and *ae* mutant line EM16 lacks amylose and has amylopectin with relatively long-unit chains [7, 8]. Its starch resists digestion *in vitro* and *in vivo* [8]. The RS content of *wx/ae* brown rice is 27.8% (Table 1-1), while that of Koshihikari brown

rice is under 2%. In addition, *wx/ae* brown rice has more dietary fiber and γ-oryzanol than Koshihikari brown rice. These three nutrients have beneficial effects on dyslipidemia and insulin resistance. The consumption of RS and dietary fiber reduces the risk of type 2 diabetes [15, 16, 18]. γ-Oryzanol has beneficial effects on hypercholesterolemia, hyperlipidemia, and insulin resistance in animal models [21, 22], and has been approved for the treatment of dyslipidemia in Japan. The results of the present study, as expected from the data of nutritional information, show that the intake of *wx/ae* brown rice resulted in better plasma lipid level and glycemic status than intake of Koshihikari brown rice by high-fat diet-fed NSY/Hos mice, type 2 diabetic mice with clinical characteristics resembling the common forms of type 2 diabetes in humans [26, 27]. The plasma total cholesterol, non-HDL cholesterol, TAG and NEFA levels, and the total cholesterol/HDL cholesterol ratio were significantly lower in the *wx/ae* group than in the Koshihikari group. The fasting blood glucose level throughout the experimental period and the pathological score of glycosuria at 10 weeks were significantly lower in the *wx/ae* group. I also observed a protective effect on high-fat diet-induced liver damage. AST and ALT, both markers for liver conditions, were lower in the *wx/ae* group. These results indicate that *wx/ae* brown rice was more effective than Koshihikari brown rice in preventing dyslipidemia, cardiovascular disease, and hyperglycemia.

Inhibiting dietary lipid absorption induces a favorable change in the plasma lipid content [33]. Gastrointestinal lipase inhibition prevents dyslipidemia [36], and inhibition of bile acid

reabsorption ameliorates hypercholesterolemia [14, 25]. I observed in this study lower levels of plasma TAG, NEFA, and fasting glucose in the *wx/ae* group, as well as a five-fold increase in the fecal TAG level. These results indicate that *wx/ae* inhibited the absorption of dietary TAG, resulting in decreased plasma TAG and NEFA levels. In addition, gastrointestinal lipase inhibition has been shown to decrease glycemia, because of the close relationship between glucose and lipid for energy metabolism [36], indicating that the increased excretion of fecal TAG may be involved in the prevention of hyperglycemia by *wx/ae* brown rice. Tsujita and colleagues have reported that basic polysaccharides could inhibit lipase activity, promote fecal lipid excretion, and reduce the plasma TAG level [37]. RS in *wx/ae* brown rice might therefore inhibit lipase activity. On the other hand, rice bran has also been shown to inhibit the pancreatic lipase activity *in vitro*, and an aqueous extract of defatted rice bran suppressed visceral fat accumulation in rats[38], suggesting that *wx/ae* brown rice might contain constituents with pancreatic lipase inhibitory activity.

Dietary RS resists digestion in the small intestine. Undigested dietary RS ferments in the large intestine, producing short-chain fatty acids [39]. Fermentation of dietary RS has increased secretion of the endogenous gut-secreted peptide hormones, glucagon-like peptide-1 (GLP-1) and peptide YY (PYY), by rodents [17]. GLP-1 and PYY are produced by the L cells of the gut and suppress the appetite [40]. Moreover, GLP-1 induces glucose-dependent insulin secretion. Therefore, maintaining substantially high plasma levels of GLP-1 and PYY

are desirable in the treatment of type 2 diabetes and obesity. *wx/ae* brown rice contains much more RS than Koshihikari brown rice, and this may explain the favorable glycemic status of the *wx/ae* group.

Treatment with hypolipidemic agents influences the expression of genes related to lipid metabolism in the liver [14, 25]. The present study also shows that *wx/ae* brown rice upregulates the genes related to fat synthesis and cholesterol synthesis in association with a reduced plasma lipid level. SREBP-1 regulates the expression of such genes related to fatty acid metabolism as FAS, while SREBP-2 regulates such cholesterologenic enzyme genes as HMG-CoAR and LDL-R [41]. The expression of the SREBP-1, FAS, SREBP-2, and LDL-R genes was significantly higher in the *wx/ae* group than in the Koshihikari group, while the expression of the HMG-CoAR gene also tended to be higher in the *wx/ae* group. These results indicate that lipogenesis in the liver of the *wx/ae* group mice was upregulated in comparison with the Koshihikari group mice, although no difference in the TAG or cholesterol content in the liver was apparent, suggesting that reduced plasma lipids induced hepatic lipogenesis in the *wx/ae* group.

CYP2R1 is the key enzyme required for 25 hydroxylation of vitamin D and plays an important role in regulating the concentration of serum 25-hydroxy vitamin D, the best indicator of vitamin D status [42, 43]. Mathieu *et al.* have reported that vitamin D deficiency may be related to the pathogenesis of type 1 and 2 diabetes [44]. The gene expression level of CYP2R1 in the liver in the present study was significantly higher in the *wx/ae*

group than in the Koshihikari group, suggesting that the favorable glycemic status of the *wx/ae* group may have resulted in upregulation of the CYP2R1 gene.

In summary, intake of *wx/ae* brown rice showed more beneficial effects to both the plasma lipid level and glycemic status than intake of Koshihikari brown rice by high-fat diet-fed NSY mice. Although the conclusions need to be confirmed by further studies, including a dose-dependent study on animals and a human intervention study, *wx/ae* brown rice has the potential for preventing a rise in plasma lipid and glucose levels.

CHAPTER II

Hypolipidemic Effects of Starch and γ-Oryzanol from *wx/ae* Double-mutant Rice in BALB/c.KOR-*Apoe*^{shl} Mice

2-1 Introduction

Japonica rice (*Oryza sativa* L.) *waxy/amylose-extender* (*wx/ae*) double-mutant line AMF18 was derived from a cross between a *waxy* (*wx*) mutant line EM21 and an *amylose-extender* (*ae*) mutant line EM16, and is defective in *starch branching enzyme IIb* (*SBE IIb*) and *granule-bound starch synthase I* (*GBSS I*) genes, resulting in its producing a unique starch of pure amylopectin with branched chains longer than those of normal rice amylopectin [9]. EM21 and EM16 generated by treating fertilized egg cells of *japonica* rice cv. Kinmaze with *N*-methyl-*N*-nitrosourea are genetically defective in GBSS I and SBE IIb, respectively [45]. The *wx/ae* rice also has high amount of γ-oryzanol [46].

Starches can be classified according to their behavior when incubated with digestive enzymes. Rapidly digestible starch is mostly amorphous and is easily digested in the small intestine. Slowly digestible starch consists of amorphous and raw starches with type A or C crystalline structure, or type B starch in granule or retrograded form[47]. Resistant starch (RS) cannot be

digested in the small intestine and is effective against hyperglycemia[48-50], hypercholesterolemia [51, 52], and fat accumulation [53]. RS can be divided into four types as follows [54]. RS_1 is physically inaccessible to digestion due to the presence of intact cell walls in grains, seeds or tubers. RS_2 is made of native starch granules that are protected from digestion by the conformation or structure of the starch granule, as in raw potato starch and high-amylose maize starch. RS_3 is made of non-granular starch-derived materials that resist digestion, usually formed during the retrogradation of starch granules. RS_4 describes a group of starches that have been chemically modified to reduce digestibility. In our previous study, We found that *wx/ae* starch granules have no amylose, type B crystalline structure, high gelatinization temperature, and very low digestibility [8]. Hence, I classify raw *wx/ae* starch as type RS_2.

γ-Oryzanol is a mixture of ferulic acid esters of plant sterols, and has anti-hyperlipidemic properties [55, 56]. It also has anti-inflammatory and antioxidant properties and attenuates the preference of dietary fat in mice [57, 58]. Most γ-oryzanol in the rice grain is located in the bran and some in the endosperm [59, 60].

Spontaneously hyperlipidemic (shl) mice developed by inbreeding Japanese wild mice (*Mus musculus molossinus*) have high concentrations of plasma and hepatic lipids due to disruption of the *apolipoprotein E* (*apoE*) gene [61, 62]. ApoE is an apolipoprotein present in all lipoproteins except low density lipoprotein (LDL) and an important ligand for lipoprotein receptors in mammalian tissue [63]. A deficiency of apoE in mice

retards triacylglycerol (TAG)-rich lipoprotein clearance and results in an accumulation of remnant-like particles in the bloodstream [61, 64, 65]. ApoE-deficient mice would make a suitable animal model of hyperlipidemia for the assessment of physiological activity of dietary factors. The BALB/c.KOR/Stm Slc-*Apoe*^{shl} (BALB/c.KOR-*Apoe*^{shl}) mouse, a congenic line developed from the shl mouse, changes plasma lipid concentrations gradually with age and does not have the apoA-I polymorphism that C57BL/6.KOR/Stm Slc-*Apoe*^{shl} mice have [64].

In chapter I, I found that intake of *wx/ae* brown rice is more effective than Koshihikari brown rice in lowering plasma lipid and glucose concentrations in high-fat diet fed NSY mice [46]. Because RS and γ-oryzanol have beneficial effects on lipid metabolism, I focused on *wx/ae* starch and γ-oryzanol as the main active components in *wx/ae* rice. The objective of the present study is to examine the effect of *wx/ae* starch and γ-oryzanol on lipid metabolism in BALB/c.KOR-*Apoe*^{shl} mice fed a high-fat and high-sucrose diet.

2-2 Materials and Methods

2-2-1 Materials

Amylose-free *waxy/amylase-extender* (*wx/ae*) double-mutant line AMF18 rice and its parental cv. Kinmaze (wild-type, WT) rice were grown in the summer of 2009 in Nara

prefecture in Japan and in the summer of 2007 in the experimental field of Osaka Prefecture University, respectively.

2-2-2 Starch granule isolation and defatting

A total of 1 kg of 10% milled rice grains of WT and *wx/ae* were soaked in 0.05% (w/w) sodium hydroxide for 16 hr. Rice grains were washed with distilled water several times until the supernatant showed neutral pH. Wet grains were homogenized using a mortar and pestle. Slurries were filtered, and starch granules passed through a 140-mesh sieve were sedimented by centrifugation at 500 *g* for 10 min. The isolated starches were washed with 1% (w/w) sodium dodecyl sulfate twice. After washing with distilled water more than 5 times, the starches were thoroughly washed with 70% (v/v) ethanol and dried in a decompression dryer at 50 °C for 3 days. Dried starches were homogenized by mortar and pestle and filtered through a 26-mesh sieve before defatting. The starches were defatted three times with 3 volumes of water-saturated *n*-butanol at 70 °C for 60 min [66], then filtered and dried in a decompression dryer at 50 °C for 3 days. Dried defatted starches were homogenized by mortar and pestle and filtered through a 26-mesh sieve before use.

2-2-3 Resistant starch content in defatted starches

Resistant starch content in defatted WT and *wx/ae* starches was measured using an RS assay kit (Megazyme, Wicklow, Ireland). This method is based on AOAC method 2002.02 and AACC method 32-40, and can give precise results at over 2% RS content.

2-2-4 γ-Oryzanol extraction from *wx/ae* rice

A total of 1 kg of *wx/ae* brown rice flour was soaked twice in three volumes of *n*-hexane at room temperature for 16 hr. The extract was then filtered and evaporated under reduced pressure to yield crude rice oil. I obtained pure rice oil by dewaxing and degumming crude rice oil according to the method of Zullaikah *et al* [67]. A γ-oryzanol rich fraction was separated from pure rice oil according to the method of Chen *et al* [68]. Briefly, rice oil dissolved in *n*-hexane was loaded onto an open column (550 mm length and 30 mm I.D.) packed with silica gel 60 (Nacalaitesque, Kyoto, Japan). The eluting solvent consisted of *n*-hexane and ethyl acetate. The γ-oryzanol rich fraction was dried out and crystallized according to the method of Zullaikah *et al* [67]. This γ-oryzanol was 95% pure according to the evaluation method using HPLC-UV system and commercial γ-oryzanol (Tokyo Chemical Industry, Tokyo, Japan) as a standard [46, 69].

2-2-5 Animals and diets

Male 7 week-old BALB/c.KOR/Stm Slc-*Apoe*^{shl} mice (Japan SLC, Hamamatsu, Japan) were fed an AIN-93M diet as an initial diet for 1 week [70], and then the mice were divided into four groups of seven based on body weight and plasma TAG concentration. The mice of each group were fed a high-fat and high-sucrose diet shown in Table 2-1 for 3 weeks [71]. The percentage of starch and γ-oryzanol in the diet were almost same as those of the diet containing 15% *wx/ae* milled rice. The control (C), γ-oryzanol (O), resistant starch (RS) and resistant

starch plus γ-oryzanol (RSO) groups were fed diets containing WT starch, both WT starch and γ-oryzanol, *wx/ae* starch, and both *wx/ae* starch and γ-oryzanol, respectively. All mice were housed individually with controlled temperature (23 ± 2 °C), humidity (60 ± 10%), and lighting (8:00-20:00) with free access to their diets and tap water during the experimental period. The body weight of each mouse was measured once per week, and its food intake was measured twice per week. The feces of each mouse was collected at day 15 to day 17, and weighed after freeze-drying. At day 22, all mice (12 weeks of age) were starved for 4 hr before excising organs under diethyl ether anesthesia and weighing their livers and kidneys. Blood samples were simultaneously obtained by their inferior vena cava. Plasma was separated by centrifugation (1,000 *g*, 5 min) immediately after collection and stored at -80 °C until analysis. All experimental procedures involving the laboratory animals were approved by the Animal Care and Use Committee of Osaka Prefecture University.

Table 2-1. Composition of experimental diets

	C (%)	O (%)	RS (%)	RSO (%)
WT starch	11.488	11.487	–	–
wx/ae starch	–	–	11.488	11.487
γ-oryzanol	–	0.006	–	0.006
Sucrose	20.000	19.999	20.000	19.999
Beef tallow	14.000	13.999	14.000	13.999
Lard	14.000	13.999	14.000	13.999
Soybean oil	2.000	2.000	2.000	2.000
α-Cornstarch	3.381	3.381	3.381	3.381
Cellulose powder	5.000	5.000	5.000	5.000
AIN-93 vitamin mix	1.000	1.000	1.000	1.000
AIN-93G mineral mix	3.500	3.500	3.500	3.500
Choline bitartrate	0.250	0.250	0.250	0.250
tert-Butylhydroquinone	0.006	0.006	0.006	0.006
L-Cystine	0.375	0.375	0.375	0.375
Casein	25.000	24.998	25.000	24.998
Total	100.000	100.000	100.000	100.000

2-2-6 Blood chemistry

Plasma triacylglycerol (TAG), total cholesterol, high-density lipoprotein (HDL) cholesterol, non-esterified fatty acids (NEFA), and glucose concentrations were measured with Triglyceride E-Test, Cholesterol E-test, HDL-cholesterol E-test, NEFA C-test and Glucose CII test kits, respectively (Wako, Osaka, Japan). Total cholesterol/HDL cholesterol ratio was calculated from the total cholesterol and HDL cholesterol concentrations.

2-2-7 Hepatic TAG and total cholesterol

Total lipids were extracted from 100 mg of liver tissues according to the method of Bligh and Dyer [72], and then dissolved in isopropanol containing 10% Triton X-100 (Sigma-Aldrich, Tokyo, Japan). Hepatic TAG concentration was measured using the L-type Wako TG·H (Wako). Hepatic total cholesterol concentration was measured by a modified Zak-Henly method [73].

2-2-8 Fecal TAG and total cholesterol

Total lipids were extracted from 50 mg of dried feces after weighing according to the method of Bligh and Dyer [72], and then dissolved in isopropanol containing 10% Triton X-100 (Sigma-Aldrich). Fecal TAG concentration was measured using an acetylacetone method [74]. Fecal total cholesterol concentration was measured by a modified Zak-Henly method [73].

2-2-9 Histopathology of liver

Livers were formalin-fixed, paraffin-embedded, and processed for hematoxylin and eosin (HE) staining. The prepared specimens were observed with an ECLIPSE LV100POL microscope (Nikon, Tokyo, Japan).

2-2-10 Measurement of mRNA levels in the liver

Total RNA was extracted using an RNeasy mini kit (Qiagen, Tokyo, Japan). cDNA was synthesized from 5 μg of extracted

RNA using a SuperScript III First-strand Synthesis System for RT-PCR (Life Technologies Japan, Tokyo, Japan), and then purified using a QIAquick PCR Purification kit (Qiagen). Quantitative real-time PCR was performed with SYBR Green Realtime PCR Master Mix -Plus- (Toyobo, Osaka, Japan) on a LineGene machine (BioFlux, Tokyo, Japan). Relative levels of target mRNA were normalized to the corresponding level of elongation factor-1α (EF-1α) mRNA. The sequences of the primers were as follows: EF-1α (Forward 5′-AGACCAGCAAATACTATGTGACCA-3′, Reverse 5′-TTCTTGGAGATACCAGCTTCAAAT-3′); sterol regulatory element-binding protein-1 (SREBP-1) (Forward 5′-TGCTCCTGTGTGATCTACTTCTTG-3′, Reverse 5′-TGTAGGAATACCCTCCTCATAGCA-3′); sterol regulatory element-binding protein-2 (SREBP-2) (Forward 5′-GTACTGTCACTGGAGTCAGGTGCT-3′, Reverse 5′-CTTCACAAAGACGCTCAAGACAAT-3′); fatty acid synthetase (FAS) (Forward 5′-GCACAGCTCTGCACTGTCTACTAC-3′, Reverse 5′-ATCCCAGAGGAAGTCAGATGATAG-3′); low density lipoprotein receptor (LDL-R) (Forward 5′-ATCTCTCAGCTCCCAGTACTCCTA-3′, Reverse 5′-AGAAGATGGACAGGAACCTCATAC-3′); 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG Co-AR) (Forward 5′-CAAAGAACTGACAGGCTTAAATGA-3′, Reverse 5′-TCCACGAGCTATATTTTCCCTTAC-3′). PCR products were evaluated by analysis of their melting curves (data not shown).

2-2-11 Short-chain fatty acids in cecal contents

The short-chain fatty acids (SCFA) content in cecal contents was determined from total cecal contents which were collected from each mouse and mixed for each group. The mixed cecal contents were weighed and their pH measured using a B-212 pH meter (Horiba, Kyoto, Japan). 100 mg samples were taken from the mixed cecal contents of each group, homogenized with ethanol and centrifuged (1000 g, 5 min). SCFA in the supernatant were measured for each group using a Fatty Acid Analysis kit (YMC, Kyoto, Japan) and an HPLC-UV system consisting of a PU-2089 pump (Jasco, Tokyo, Japan), an AS-2057 (Jasco), a YMC-Pack FA column (250 mm x 4.6 mm I.D., YMC), a CSL-300C column oven (Chromato Science, Osaka, Japan), and an SPD-10A UV-VIS detector (Shimadzu, Kyoto, Japan). The conditions were as follows: flow rate 1.0 mL/min, oven temperature 40 °C, injection volume 5 μL, detection UV at 230 nm, mobile phase methanol/acetonitrile/MilliQ water (16/30/54, v/v/v, pH 4.5 adjusted by 0.01 N HCl). The contents were analyzed for SCFA such as acetic acid, propionic acid and *n*-butyric acid using commercial reagents (Wako) as standards. Each analysis was performed in triplicate and coefficients of variation for the three SCFA of each group were under 15%. The cecal content weight per mouse was calculated as the average over the mice in its group, and similarly for the cecal SCFA content, but I could not obtain the standard error of these average values.

2-2-12 Statistical analyses

Except for cecal content weight and pH, and cecal SCFA content, results are given as means ± standard deviation (SD). Two-way ANOVA with a *post hoc* analysis by Tukey's test was carried out with the SAS 9.1 software program (SAS Institute, Cary, USA).

2-3 Results

2-3-1 Effects of *wx/ae* starch and γ-oryzanol on body weight and food intake

To determine the effects of *wx/ae* rice starch and γ-oryzanol on lipid metabolism, I prepared defatted starch (*wx/ae* starch) and extracted γ-oryzanol from *wx/ae* rice. Defatted starch was also prepared from WT rice (WT starch) as a control rice starch sample. The *wx/ae* starch contained 53% RS, while the WT starch contained less than 2% RS.

Four groups of mice were fed the high-fat and high-sucrose diets shown in Table 2-1 for 3 weeks. The four experimental diets contained WT starch, WT starch plus γ-oryzanol, *wx/ae* starch and *wx/ae* starch plus γ-oryzanol. All animals were in good health throughout the experiment (data not shown). As shown in Table 2-2, no significant differences in body weight gain, food intake, liver weight, or kidney weight were observed between groups. Hence, *wx/ae* starch and γ-oryzanol are not effective for obesity prevention.

Table 2-2. Physiological parameters and blood chemistry

	C	O	RS	RSO
Number	7	7	7	7
Intake (g/d)	4.0 ± 0.8	4.2 ± 0.8	3.8 ± 0.6	3.9 ± 0.4
Initial body weight (g)	22.6 ± 0.7	22.3 ± 1.7	22.6 ± 0.6	22.6 ± 1.1
Final body weight (g)	27.0 ± 1.5	27.4 ± 1.1	26.7 ± 1.4	27.3 ± 1.7
Body weight gain (g)	4.4 ± 1.1	5.2 ± 1.3	4.1 ± 1.2	4.7 ± 1.3
Kidney weight (mg)	491 ± 30	522 ± 41	485 ± 37	487 ± 27
Liver weight (g)	1.48 ± 0.17	1.50 ± 0.17	1.37 ± 0.13	1.39 ± 0.13
Hepatic TC (mgl/g)	12.3± 3.6	10.8± 2.5	12.2± 1.6	10.4± 3.1
Dried feces (mg/d)	301 ± 26 ^{b}	299 ± 50 ^{b}	469 ± 104 ^{a}	478 ± 43 ^{a}
Fecal lipid excretion				
TAG (mg/d)	2.94 ± 0.51^{b}	2.45 ± 0.39^{b}	3.75 ± 1.15^{ab}	4.64 ± 1.45^{a}
Total cholesterol (mg/d)	2.57 ± 0.45	2.29 ± 0.44	2.38 ± 0.61	2.67 ± 0.21
Blood chemistry				
Total cholesterol (mg/dL)	611 ± 67	567 ± 57	528 ± 128	498 ± 96
HDL-cholesterol (mg/dL)	114 ± 41	151 ± 43	138 ± 47	144 ± 54
Total cholesterol/HDL-cholesterol ratio	5.8 ± 1.8	3.9 ± 0.8	4.2 ± 1.8	3.9 ± 1.8
NEFA (mEq/L)	2.2 ± 1.0	1.8 ± 0.9	1.6 ± 0.3	1.3 ± 0.4
Glucose (mg/dL)	281 ± 44	281 ± 43	267 ± 64	269 ± 56

Each value is expressed as means ± SD.

Means not sharing a superscript letter differ significantly ($P < 0.05$).

2-3-2 Hypolipidemic effects by combining *wx/ae* starch and γ-oryzanol

To examine the effect of *wx/ae* starch and γ-oryzanol on lipid metabolism, I measured plasma lipid concentrations (Table 2-2 and Figure 2-1). There were no significant differences in the blood chemistry data tested among the C, O, and RS groups, while the TAG concentration in the RSO group was significantly lower than that of the C ($P < 0.001$), O ($P < 0.005$) and RS ($P <$

0.05) groups. Moreover, total cholesterol (P = 0.12) and NEFA (P = 0.11) concentrations and the total cholesterol/HDL cholesterol ratio (P = 0.14) in the RSO group tended to be lower than those in the C group. There were no significant differences in HDL cholesterol and glucose concentrations in the RSO group.

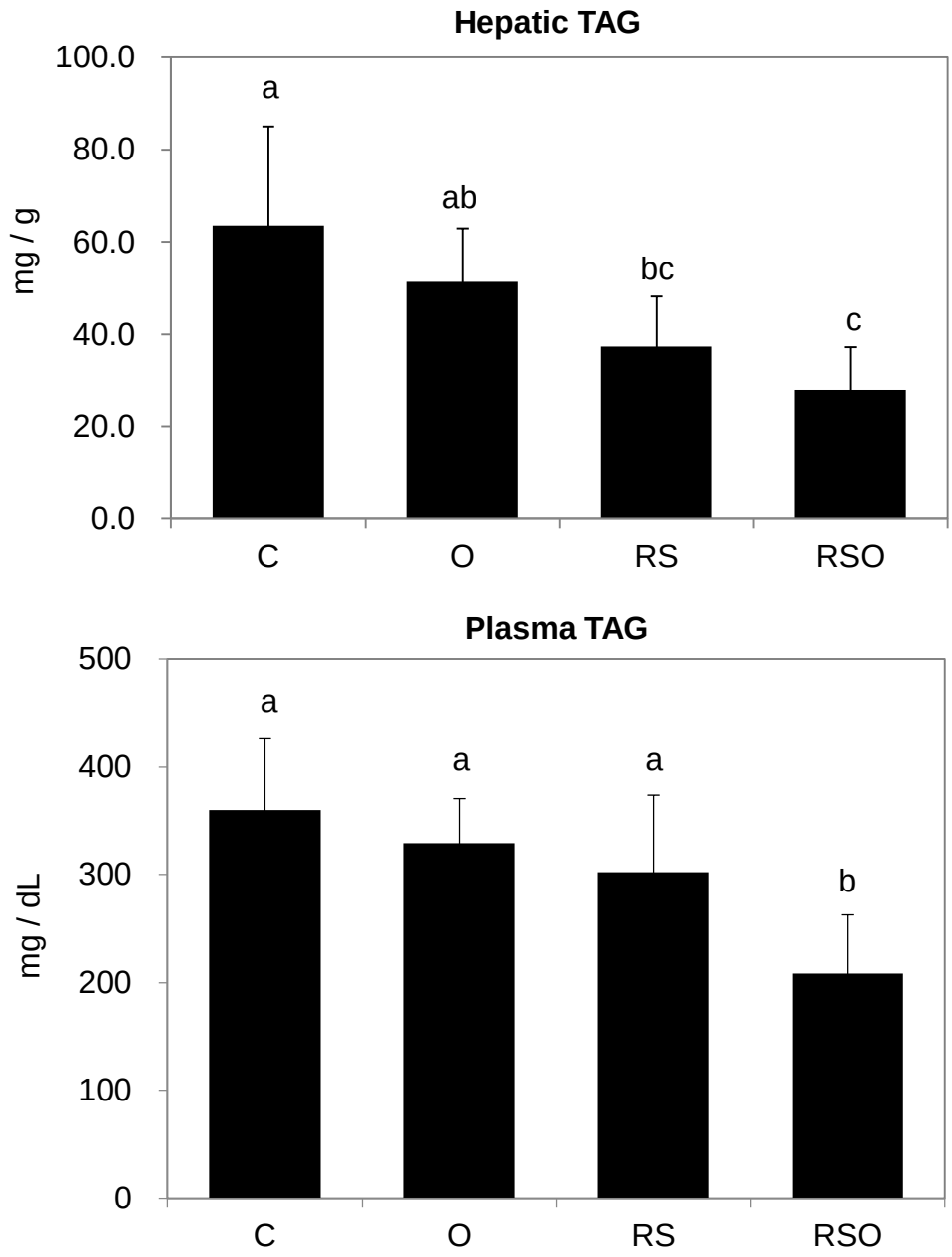


Figure 2-1. TAG concentrations in plasma and liver.

Results represent means ± SD (n = 7). Means not sharing a superscript letter differ significantly ($P < 0.05$).

2-3-3 Prevention of fatty liver by combining *wx/ae* starch and γ-oryzanol

As shown in Table 2-2 and Figure 2-1, there were no differences in liver weight between groups, but TAG concentration in the liver was significantly lower in the RS ($P < 0.05$) and RSO groups ($P < 0.001$) than in the C group, and the O and RSO groups were significantly different from each other ($P < 0.05$). To investigate this lowered TAG concentration in the liver, I used hematoxylin and eosin (HE) staining (Figure 2-2). I observed no fibrosis or inflammation in any group, but hepatocytes in the centrilobular area were rich in unstained cytoplasm in the C and O groups than in the RS and RSO groups, indicating that fatty liver was prevented in the latter.

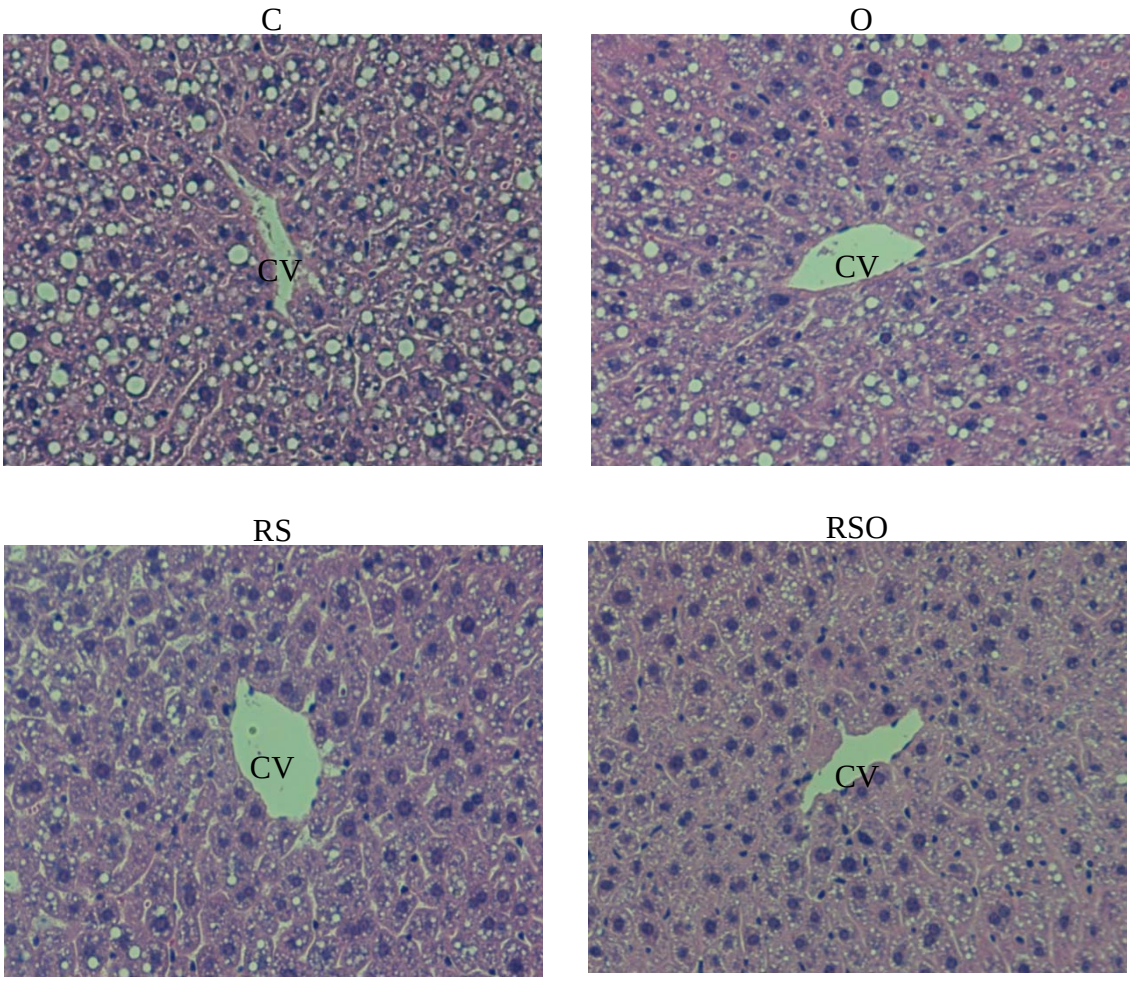


Figure 2-2. Histopathology of liver tissues (HE staining).
CV: central vein. Magnification: x400.

2-3-4 Gene expression in the liver

Plasma and hepatic TAG concentrations were significantly lower in the RSO group than in the C group. To determine the effects of *wx/ae* starch and γ-oryzanol on lipid metabolism, I performed real-time RT-PCR analysis on the liver (Figure 2-3). Among the six genes tested, only the SREBP-1 gene was

significantly downregulated in the O ($P < 0.01$), RS ($P < 0.005$), and RSO groups ($P < 0.001$). I found slight decreases in the SREBP-2, HMG-CoAR and LDL-R genes in the O, RS and RSO groups, but nothing of statistical significance in the other five genes.

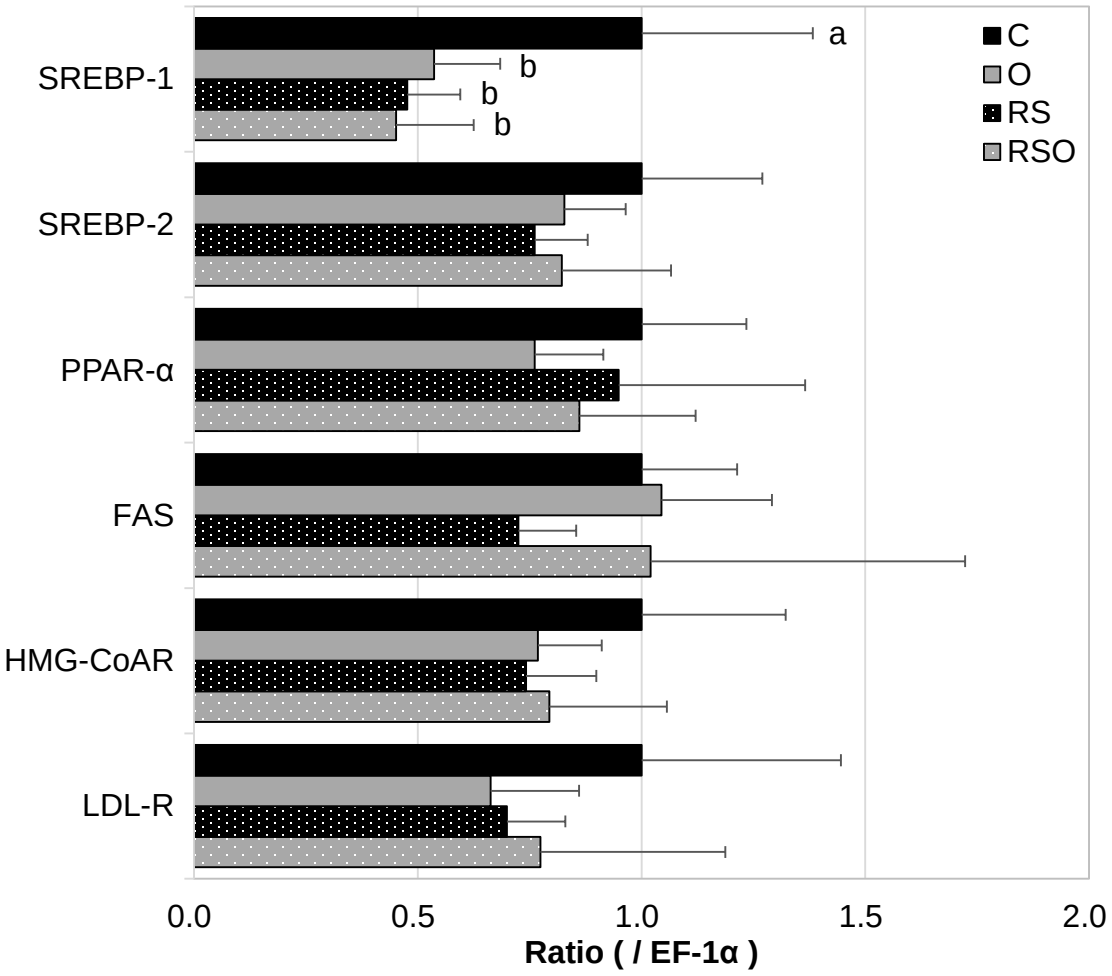


Figure 2-3. Gene expression analysis in the liver by SYBR green-based real-time RT-PCR.

EF-1α was used as a standard to normalize the efficiency of each reaction, and results (mean ± SD; $n = 7$) are presented relative to the C group. Means not sharing a superscript letter differ significantly ($P < 0.05$).

2-3-5 Effects on fecal weight and lipids

Inhibiting dietary lipid absorption can lower the plasma lipid concentrations. I therefore analyzed fecal lipid excretion to study the effect of *wx/ae* starch and γ-oryzanol on dietary lipid absorption (Table 2-2). Dried fecal weight was significantly higher in the RS and RSO groups ($P < 0.001$) after 3 weeks' treatment, but a significant increase in fecal TAG excretion was observed only in the RSO group ($P < 0.05$ compared with the C group and $P < 0.005$ compared with the O group). There was no significant difference between groups in total cholesterol excretion. These results indicate that *wx/ae* starch and γ-oryzanol show synergistic effects in inhibiting dietary TAG absorption.

2-3-6 Effects on short-chain fatty acid content in the cecum

Dietary RS increases the content weight and SCFA content in the cecum. SCFA have beneficial effects on intestinal functions and lipid and carbohydrate metabolism. I measured SCFA content in the cecum to characterize the changes in intestinal environment made by *wx/ae* starch and γ-oryzanol. Table 2-3 shows average values per mouse of cecal content weight and pH, and cecal SCFA content. Cecal content weight and the content of the three SCFA in the RS and RSO groups tended to be higher than those in the C group, while there were no differences between the C and O groups. Cecal content pH in the RS and RSO groups tended to be lower than that in the C group. These results suggest that γ-oryzanol does not affect cecal content weight and SCFA content, and the fermentation of

wx/ae starch produces SCFA, including acetic acid, propionic acid and *n*-butyric acid. I note that significant differences in the results should be determined by statistical significance tests in further experiments taking into account differences between individual mice.

Table 2-3. Average values per mouse of cecal content weight and pH, and cecal SCFA content

	C	O	RS	RSO
Cecal content weight (mg/mouse)	102	157	250	243
Cecal content pH [a)]	7.7	7.4	7.1	7.2
Cecal SCFA content [a)]				
Acetic acid (μmol/mouse)	5.6	6.8	15.7	11.7
Propionic acid (μmol/mouse)	0.9	1.0	2.8	2.6
n-Butyric acid (μmol/mouse)	0.5	0.6	3.0	2.8

[a)] Average values for three measurements.

2-4 Discussion

wx/ae double-mutant *japonica* rice characteristically produces RS and high amount of γ-oryzanol. In chapter I, I found that *wx/ae* brown rice is more effective than cv. Koshihikari brown rice in reducing both plasma lipid and glucose concentrations in high fat diet-fed NSY mice [46]. In this chapter, I prepared defatted starch (RS content 53%) and γ-oryzanol from *wx/ae* rice and fed them to ApoE-deficient mice to study the role

of *wx/ae* starch and γ-oryzanol in the hypolipidemic effects of *wx/ae* rice. γ-Oryzanol at a concentration of 0.006% in the diet had no effect on lipid metabolism, and *wx/ae* starch (11.488%) prevented accumulation of TAG in the liver. Intake of both *wx/ae* starch and γ-oryzanol was significantly effective in preventing not only TAG accumulation in the liver, but also the rise in plasma TAG concentration, indicating that *wx/ae* starch and γ-oryzanol may have synergistic beneficial effects on lipid metabolism.

Intake of resistant starch or γ-oryzanol affects gene expression in the liver [75-77]. The present study shows that *wx/ae* starch and γ-oryzanol downregulated the genes related to fatty acid synthesis and cholesterol synthesis. SREBP-1 is known to regulate the expression of genes like FAS that are related to fatty acid metabolism, while SREBP-2 regulates such cholesterologenic enzyme genes as HMG-CoAR and LDL-R[78]. Expression of the SREBP-1 gene was significantly lower in the O, RS and RSO groups than in the C group, and expression of the SREBP-2, HMG-CoAR and LDL-R genes tended to be lower in the O, RS and RSO groups than in the C group, indicating that lipogenesis in the liver of the O, RS and RSO groups was downregulated in association with reduced plasma and hepatic lipid concentrations.

Dietary RS increases fecal weight and the amount of cecal SCFA [54]. In this chapter, I observed that fecal weight and fecal TAG excretion were higher and that the cecal SCFA content tended to be higher in the groups fed *wx/ae* starch than in the groups fed WT starch, indicating that *wx/ae* starch exerts a

biological effect as dietary RS. *wx/ae* starch probably inhibits TAG absorption in the small intestine by adsorbing lipids itself. SCFA, especially propionic acid, inhibits lipogenesis in the liver [79, 80]. Therefore, prevention of fatty liver in the RS and RSO groups might in part depend on amount of produced SCFA. SCFA also induces Glucagon-like peptide-1 and peptide YY which improve insulin secretion and have antidiabetec effects [17]. Although I observed no beneficial effect on glycemic status by intake of *wx/ae* starch, my results in chapter I showed that intake of *wx/ae* brown rice prevented a rise in blood glucose concentration and glycosuria level [46], suggesting that produced SCFA might have some beneficial effect on glycemic status in mice fed *wx/ae* starch.

Cicero and Gaddi noted that dietary γ-oryzanol decreases plasma total cholesterol, LDL-cholesterol and TAG and increases HDL cholesterol in mammals, including rodents [55]. Because the amount of γ-oryzanol I fed my mice was much smaller than that used in chapter I [46], I observed no difference in plasma lipid concentrations between the C and O groups. It is also unclear if the antioxidant properties are due to the addition of *tert*-butylhydroquinone in the diet. However, the combination of *wx/ae* starch and γ-oryzanol partially inhibited the rise in plasma and hepatic TAG concentrations. This suggests that a small amount of γ-oryzanol can enhance hypolipidemic effects promoted by RS.

In summary, *wx/ae* starch in the diet prevented fatty liver and *wx/ae* starch supplemented with a small amount of γ-oryzanol was more effective than *wx/ae* starch alone. The combination of

wx/ae starch and γ-oryzanol had a synergistic hypotriglyceridemic effect on plasma and liver in BALB/c.KOR-*Apoe*^{shl} mice fed a high-fat and high-sucrose diet. Therefore, rice containing a large amount of RS and γ-oryzanol, such as *wx/ae* rice, has the potential for preventing fatty liver and hypertriglyceridemia.

CHAPTER III

Hypotriacylglycerolemia Effects of Starch and γ-Oryzanol from *wx/ae* Double-mutant Rice in the Liver of BALB/c.KOR-*Apoe*^{shl} Mice

3-1 Introduction

Triacylglycerols (TAGs) consist of a glycerol backbone esterified with saturated or unsaturated fatty acids (FAs), or a mixture of both. TAGs supply free FAs, an important energy source as nutrients or materials for other lipids such as cholesterol ester, phosphatidylcholine and phosphatidyl ethanolamine [81-83]. Free FAs also act as signaling molecules in various cellular processes [84-87].

Current analytical methods for TAGs include commercial kits using lipoprotein lipase (LPL) to hydrolyze the fatty acids from the TAGs [88-90]. These methods can give total TAG content but cannot give the content of specific TAG molecular species, because they nonspecifically analyze the fatty acids released by LPL. Thus, I prepared an alternative approach that uses a two-dimensional high performance liquid chromatography (2D HPLC) system coupled with two variable wavelength detectors (VWDs) and a charged aerosol detector (CAD) for detailed analysis of TAGs. The charged aerosol detection method

involves nebulizing the HPLC column effluent, evaporating the solvents, charging the aerosol particles and measuring the current form of the charged aerosol flux [91, 92]. The major advantages of the CAD are its low minimum limits of detection and its nearly linear mass-to-peak area relationship for many types of lipids [93]. This 2D HPLC system is equipped with normal phase chromatography in the first-dimension and reverse phase chromatography in the second-dimension, and provides quantitative analysis that focuses on TAG molecular species from a mixture of lipids.

In chapter II, I found that *wx/ae* starch and γ-oryzanol had a hypotriacylglycerolemia effect on liver in BALB/c.KOR-*Apoe*^{shl} mice fed a high-fat and high-sucrose diet [90]. I focused on a TAG molecular species in the liver of those mice. The objective of the present study is to examine the change of TAG molecular species content in the liver of BALB/c.KOR-*Apoe*^{shl} mice fed a high-fat and high-sucrose diet by intake of starch and γ-oryzanol from *wx/ae* mutant rice.

3-2 Materials and Methods

3-2-1 Total lipids extraction from liver

Liver tissues were obtained from BALB/c.KOR-*Apoe*^{shl} mice fed a high-fat and high-sucrose diet including starch and γ-oryzanol from *wx/ae* mutant rice, described in chapter 2. Briefly, BALB/c.KOR-*Apoe*^{shl} mice (Japan SLC, Hamamatsu,

Japan) were divided into four groups of seven based on body weight and plasma TAG concentration. The mice of each group were fed a high-fat and high-sucrose diet shown in Table 2-1 for 3 weeks [71]. The control (C), γ-oryzanol (O), resistant starch (RS) and resistant starch plus γ-oryzanol (RSO) groups were fed diets containing WT starch, both WT starch and γ-oryzanol, *wx/ae* starch, and both *wx/ae* starch and γ-oryzanol, respectively. At day 22, all mice (12 weeks of age) were starved for 4 hr before excising organs. The liver tissues were stored at -80 °C immediately after collection until extraction. All experimental procedures involving the laboratory animals were approved by the Animal Care and Use Committee of Osaka Prefecture University.

3-2-2 Two-dimensional high performance liquid chromatography

TAG molecular species were measured from total lipid extract by using a two-dimensional high performance liquid chromatography (2D HPLC) system from total lipid extract. The system consisted of an Ultimate 3000 Standard LC System equipped with a quaternary pump, an autosampler, a column oven, two variable wavelength detectors (VWD) and a Corona Charged Aerosol Detector (CAD) (Thermo Fisher Scientific, US), a six-port two-position auto valve and an LC-10AD pump (Shimadzu, Kyoto, Japan) (Figure 3-1). The conditions of the first-dimension of the 2D HPLC system were as follows: gradient mobile phase of hexane/tert-butylmethylether/methanol, 1.0 ml/min flow rate, a PVA-Sil column for a first dimension column

(250 mm x 4.6 mm I.D., YMC, Kyoto, Japan), 30 °C oven temperature, 10 µl injection volume, UV detection at 210 nm. The conditions of the second–dimension of the 2D HPLC system were as follows: mobile phase of acetonitrile/acetone (30/70, v/v), 1.0 ml/min flow rate, a Cadenza CL-C18 column (250 mm x 4.6 mm I.D., Imtakt, Kyoto,Japan), 30 °C oven temperature, detection by UV at 210 nm and CAD. The first- and second-dimensions were connected by a trap system, using a six-port two-position auto valve equipped with a YMC-Pack ProC18RS column (50 mm x 4.6 mm I.D., YMC) and an LC-10AD pump. The tarp conditions were as follows: gradient mobile phase of methanol/ethanol/MilliQ water, 5.0 ml/min flow rate. The Chromeleon software program (Thermo Fisher Scientific) was used for system control and data analysis.

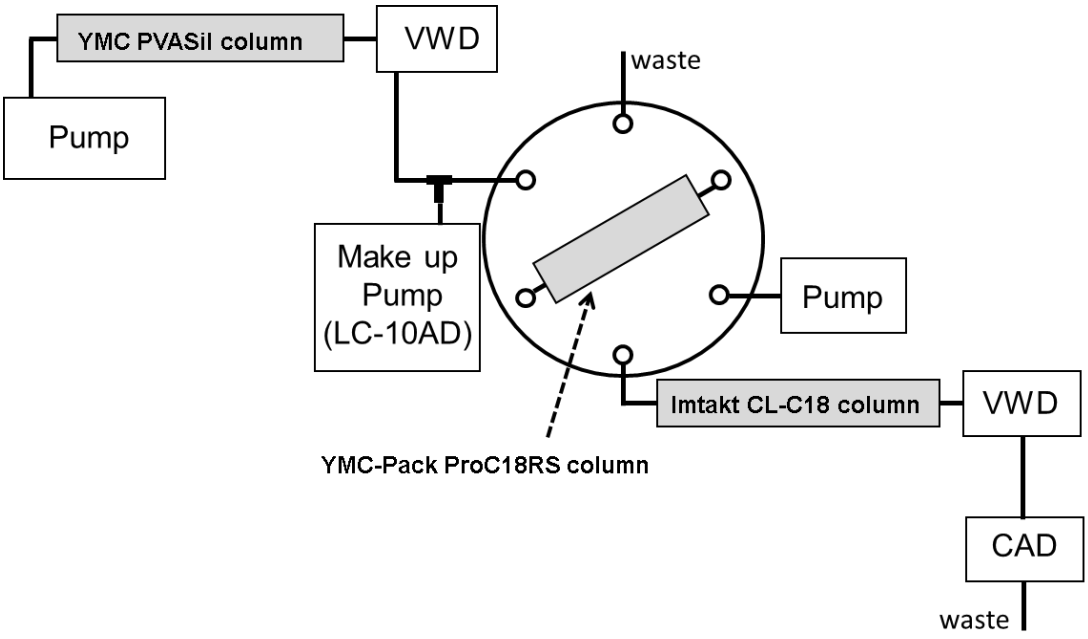


Figure 3-1. Flow diagram of 2D-HPLC system.

3-2-3 Identification of fatty acid composition of triacylglycerol molecular species

To identify the FA composition of characteristic TAG molecular species on the 2D HPLC chromatogram, I prepared a preparative 2D HPLC system. This system is based on 2D HPLC and has a fractionation line that splits from the analytical line before CAD. The fractions of certain peaks were collected using the preparative 2D HPLC. TAG molecular species in the fractions were hydrolyzed and methylated using a Fatty Acid Methylation Kit (Nacalai Tesque, Kyoto, Japan), and fatty acid methyl esters (FAME) were analyzed by using a GCMS QP-2010 Ultra (Shimadzu) with a DB-225 column (30 m length, 0.25 mm I.D, 0.15 μm film, Agilent Technologies, Santa Clara, US). 1 μl of each Samples were injected into the GC injector at 240 °C and operated in splitless mode. Helium gas with 99.999% purity was used as the carrier gas at a flow rate of 10.0 ml/min. The initial temperature was set at 35 °C for 0.5 min. The subsequent temperature was programmed at a heating rate of 25 °C/min to 195 °C, at 3 °C/min to 205 °C and at 8 °C/min until 235 °C and kept for 1 min. The ion source temperature was 200 °C. Mass units were monitored from 35 to 400 *m/z*, the components of FAME were identified by comparison of their retention time and mass spectra with a NIST mass spectral library. A F.A.M.E. Mix C14-C22 (SUPELCO, Bellefonte, US) was used as a standard.

3-2-4 Statistical analyses

Results are given as means ± standard deviation (SD). Two-way ANOVA with a *post hoc* analysis by Tukey's test was

carried out with the SAS 9.3 software program (SAS Institute, Cary, USA).

3-3 Results and discussion

I separated the TAG species by 2D HPLC analysis using livers from BALB/c.KOR-*Apoe*^{shl} mice fed starch and γ-oryzanol from *wx/ae* double mutant rice. Figure 3-2 shows the TAG profile of each group. I observed some major peaks and many minor peaks in their chromatograms and no dramatic change between each group. I recognized 62 peaks and determined five characteristic peaks from the chromatogram of each mouse (Table 3-1). Relative peak area and differences in each chromatogram relative to that of the C group are shown in Figure 3-3 A and B.

Most TAG molecular species in the mouse liver are composed of one palmitic acid and two oleic acids (POO), or a palmitic acid, an oleic acid and a linoleic acid (POL). The POL content of the RS groups was higher than that of the C group (P = 0.03), but that of the O and RSO groups were no different from that of the C group (P = 0.14 and P = 0.77, respectively). The POO content of the four groups was not significantly different. The OOO content of the RS and RSO groups was lower than that of the C group (P = 0.03 and P = 0.07, respectively), but there was no difference between the C and O groups. The OOL content of the RS group was lower than that of the C group (P = 0.08),

but that of the O and RSO groups showed no significant differences compared with that of the C group. I also observed that the SOO content of RSO groups was higher than that of the C group (P = 0.06), but that of the O and RS groups showed no difference. These results suggest that RS and γ-oryzanol affected the TAG molecular species ratio in the liver of apoE-deficient mice.

TAGs supply FAs to cholesterol ester, phosphatidylcholine, phosphatidyl ethanolamine and the free FA pool [81, 82]. Lipids containing polyunsaturated fatty acids become oxidized lipids because polyunsaturated fatty acids, like oleic acid and the linoleic acid are autoxdized easily [94, 95]. Oxidized lipids cause DNA damage and arteriosclerosis [96, 97]. Furukawa *et al.* reported that oleic acid increased TAG synthesis and stimulated TAG secretion in HepG2 cells, a suitable *in vitro* model system for the study of polarized human hepatocytes [98]. I observed that the content of TAGs composed of polyunsaturated fatty acids, OOO and OOL, was lower in the O, RS and RSO groups than in the C group. It could be that the mice fed RS and/or γ-oryzanol avoid damage from oxidized lipids and TAG synthesis and secretion.

In summary, *wx/ae* starch and γ-oryzanol in the diet caused not only a quantitative but also a qualitative change in TAGs in the liver of BALB/c.KOR-*Apoe*^{shl} mice. The relationship between the qualitative change and lipid metabolism is unclear, but I suspect that a qualitative change of TAG would be an important sign of improved lipid metabolism.

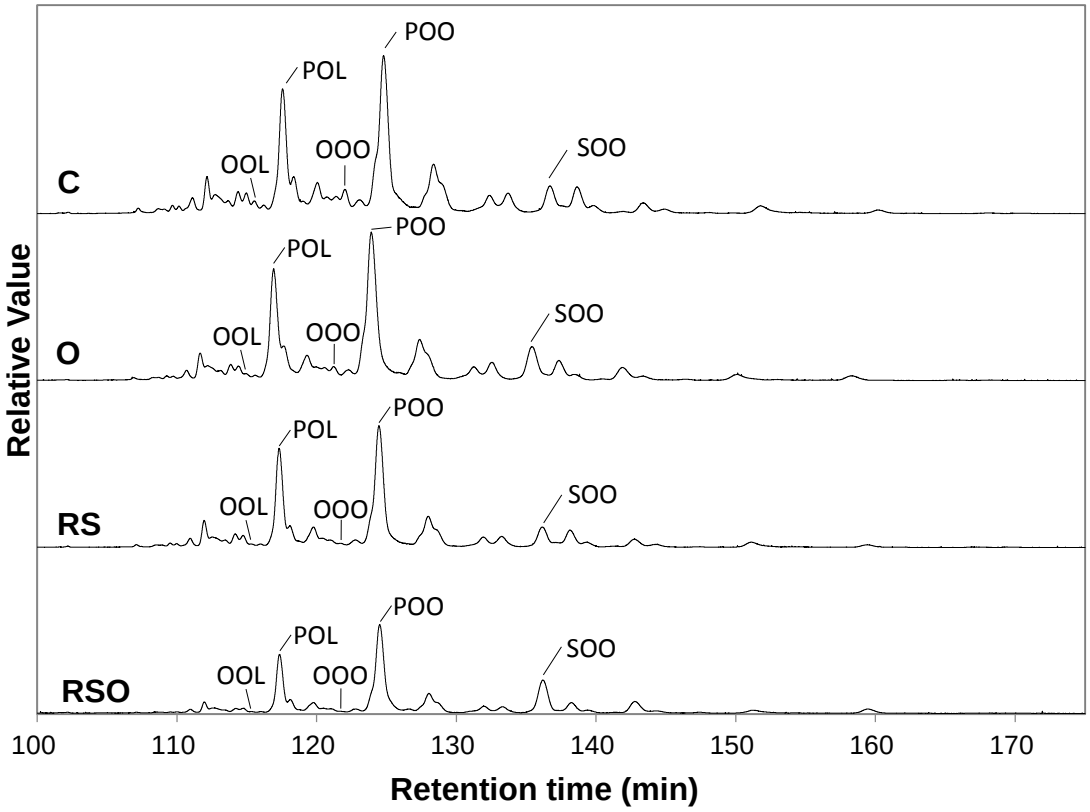


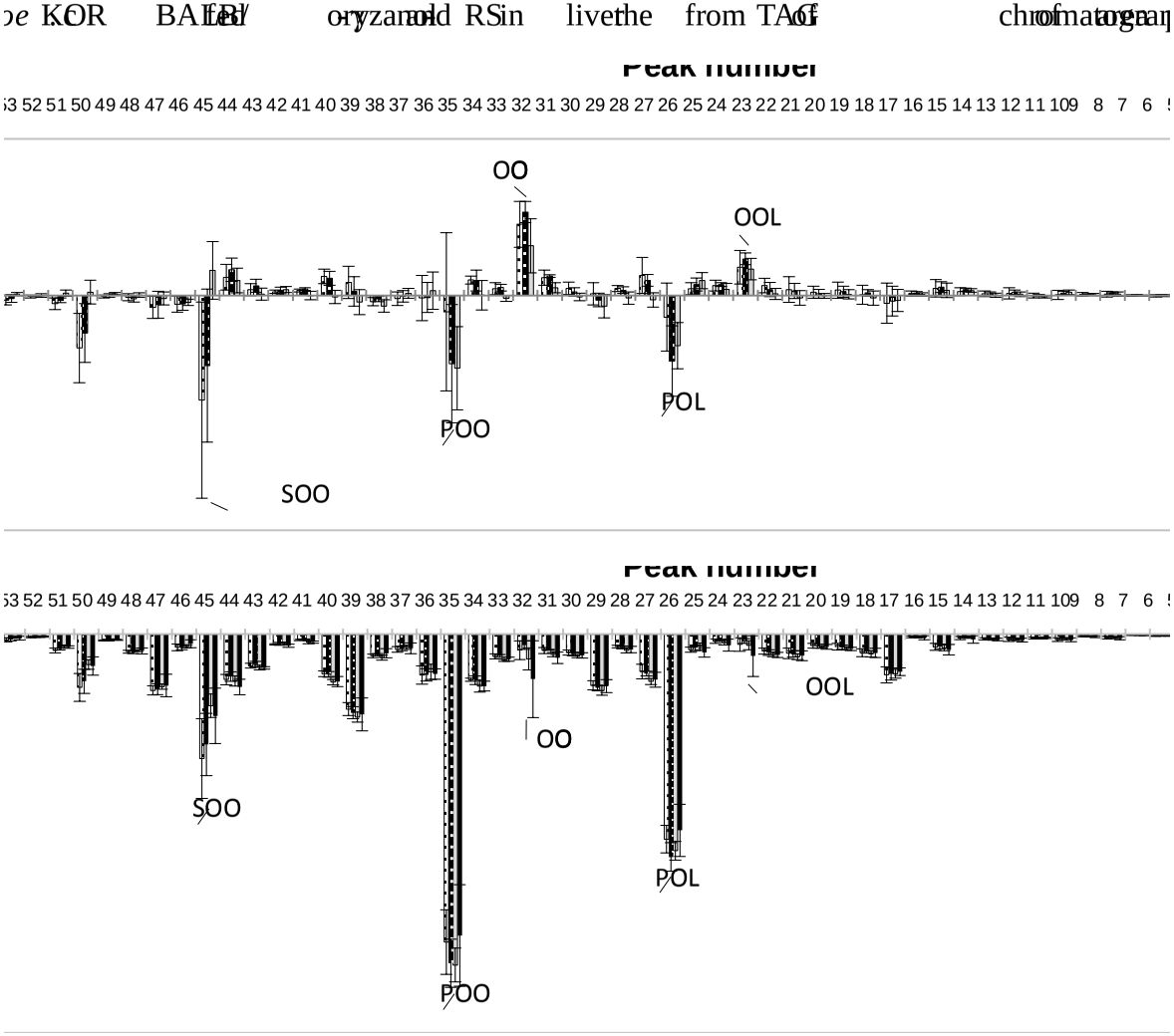
Figure 3-2. HPLC-CAD chromatograms of TAG from the liver in RS- and γ-oryzanol- fed BALB/c.KOR-*Apoe*^{shl} mice.

Table 3-1. FA comosition and peak area (%) of major TAG molecular species

Peak No.	FA composition	C	O	RS	RSO
22	OOL	1.4 ± 1.3	0.7 ± 0.3	0.4 ± 0.2	0.6 ± 0.4
25	POL	12.3 ± 1.6 [b]	13.6 ± 0.6 [ab]	14.0 ± 0.9 [a]	12.9 ± 0.9 [ab]
31	OOO	2.8 ± 2.4 [a]	1.6 ± 0.7 [ab]	0.7 ± 0.3 [b]	1.0 ± 0.6 [ab]
34	POO	18.9 ± 3.2	20.8 ± 1.1	20.7 ± 1.5	19.3 ± 2.0
44	SOO	5.1 ± 1.7 [ab]	4.5 ± 0.7 [b]	6.9 ± 2.0 [ab]	7.8 ± 2.5 [a]

Each value is expressed as the mean ± SD, (n = 7).

Means not sharing a superscript letter differ significantly (P < 0.05).


Peak number
53 52 51 50 49 48 47 46 45 44 43 42 41 40 39 38 37 36 35 34 33 32 31 30 29 28 27 26 25 24 23 22 21 20 19 18 17 16 15 14 13 12 11 10 9 8 7 6 5
OO
OOL
POL
POO
SOO
Peak number
53 52 51 50 49 48 47 46 45 44 43 42 41 40 39 38 37 36 35 34 33 32 31 30 29 28 27 26 25 24 23 22 21 20 19 18 17 16 15 14 13 12 11 10 9 8 7 6 5
OOL
OO
SOO
POL
POO

CONCLUSION

This study shows the hypolipidemic effects of eating *waxy/amylose extender* (*wx/ae*) double mutant rice and its components, resistant starch and γ-oryzanol, in two types of lifestyle-related disease model mice. Resistant starch (RS) and γ-oryzanol in the diet caused both quantitative and qualitative change in TAGs in the liver of BALB/c.KOR-*Apoe*^{shl} mice.

First, intake of *wx/ae* brown rice showed more beneficial effects on both plasma lipid level and glycemic status than intake of Koshihikari brown rice in high-fat diet-fed type 2 diabetes model NSY/Hos mice. I concluded that *wx/ae* brown rice has the potential for preventing a rise in plasma lipid and glucose levels.

I then focused on *wx/ae* starch and γ-oryzanol as the main active components in *wx/ae* rice and examined hypolipidemic effects in hyperlipidemia model BALB/c.KOR-*Apoe*^{shl} mice. *wx/ae* starch in the diet prevented fatty liver, and *wx/ae* starch supplemented with a small amount of γ-oryzanol was more effective than *wx/ae* starch alone. The combination of *wx/ae* starch and γ-oryzanol had a synergistic hypotriglyceridemic effect in the plasma and liver in BALB/c.KOR-*Apoe*^{shl} mice fed a high-fat and high-sucrose diet.

In addition, I observed that *wx/ae* starch and γ-oryzanol in the diet caused not only a quantitative but also a qualitative change in TAGs in the liver of BALB/c.KOR-*Apoe*^{shl} mice. The relationship between the qualitative change and lipid metabolism

is unclear, but I suspect that a qualitative change of TAG would be an important sign of improved lipid metabolism.

As stated above, *wx/ae* rice and its components, RS and γ-oryzanol, have beneficial effects on hyperlipidemia in two types of lifestyle-related disease model mice. Although the conclusions need to be confirmed by further studies, including a human intervention study, *wx/ae* rice and its components, resistant starch and γ-oryzanol, have the potential for improving lipid metabolisms in the plasma and liver.

ACKNOWLEDGEMENTS

The author gratefully acknowledge the following people.

First of all, I wish to thank my supervisor, Dr. Shinichi Kitamura, Professor of Osaka Prefecture University, for his support, guidance and assistance throughout this study.

I would like to thank Dr. Shinji Tanimori, Professor of Osaka Prefecture University, and Dr. Ryoichi Yamaji, Professor of Osaka Prefecture University, for their critical review and kind guidance on this dissertation.

I express my sincere thanks Dr. Kenji Matsumoto, Associate Professor of Ishikawa Prefectural University, for his support, valuable suggestions, kind guidance and advice on this study.

I would especially like to thank Dr. Hikaru Satoh, Specially Appointed Professor of Kyushu University for providing rice seeds and valuable suggestions.

I wish to acknowledge Dr. Aiko Shojo, Lecturer of Soai University, Dr. Hiroaki Hirai, Lecturer of Osaka Prefecture University, and Ms. Hatsuho Takemitsu, International Polysaccharide Engineering Inc., for their kind suggestions and encouragement.

I also want to thank Ms. Miyako Kotaniguchi, Dr. Joseph J. Rodrigue and Dr. Kanako Yoshizawa, Laboratory of Biophysical Chemistry of Osaka Prefecture University, and Dr. Shiho Suzuki, International Polysaccharide Engineering Inc., and for their

valuable help.

Special thanks are also due to all the past and present members of the Laboratory of Biophysical Chemistry of Osaka Prefecture University.

Finally yet importantly, I would like to give my special thanks to my family, especially my son, Hugh, for their support and encouragement during my study.

LIST OF PUBLICATIONS

1. Kenji Matsumoto, Masashi Maekawa, Makoto Nakaya, Hatsuho Takemitsu, Hikaru Satoh, Shinichi Kitamura: **Wx/ae Double-Mutant Brown Rice Prevents the Rise in Plasma Lipid and Glucose Levels in Mice.** *Bioscience Biotechnology and Biochemistry* 2012, **76:**2112-2117.

2. Makoto Nakaya, Aiko Shojo, Hiroaki Hirai, Kenji Matsumoto, Shinichi Kitamura: **Hypolipidemic effects of starch and γ-oryzanol from wx/ae double-mutant rice on BALB/c.KOR-Apoe(shl) mice.** *Bioscience Biotechnology and Biochemistry* 2013, **77:**1435-1440.

REFERENCES

1. Daar AS, Singer PA, Persad DL, Pramming SK, Matthews DR, Beaglehole R, Bernstein A, Borysiewicz LK, Colagiuri S, Ganguly N, et al: **Grand challenges in chronic non-communicable diseases.** *Nature* 2007, **450:**494-496.

2. Muratani H, Kimura Y, Fukiyama K, Kawano Y, Fujii J, Abe K, Kuwajima I, Ishii M, Shiomi T, Mikami H, et al: **Control of blood pressure and lifestyle-related risk factors in elderly Japanese hypertensive subjects.** *Hypertension Research* 2000, **23:**441-449.

3. Wada K, Sakamoto H, Nishikawa K, Sakuma S, Nakajima A, Fujimoto Y, Kamisaki Y: **Life style-related diseases of the digestive system: endocrine disruptors stimulate lipid accumulation in target cells related to metabolic syndrome.** *Journal of Pharmaceutical Sciences* 2007, **105:**133-137.

4. Parekh S, Anania FA: **Abnormal lipid and glucose metabolism in obesity: implications for nonalcoholic fatty liver disease.** *Gastroenterology* 2007, **132:**2191-2207.

5. Chang YY, Chiou WB: **Taking weight-loss supplements may elicit liberation from dietary control. A laboratory**

experiment. *Appetite* 2014, **72:**8-12.

6. Zyoud SH, Abd-Alhafez AB, Hussein AO, Abu-Shehab IS, Al-Jabi SW, Sweileh WM: **Patterns of use of medications, herbal products and nutritional supplements and polypharmacy associating factors in Palestinian geriatric patients.** *European Geriatric Medicine* 2013.

7. Kubo A, Yuguchi Y, Takemasa M, Suzuki S, Satoh H, Kitamura S: **The use of micro-beam X-ray diffraction for the characterization of starch crystal structure in rice mutant kernels of waxy, amylose extender, and sugary1.** *Journal of Cereal Science* 2008, **48:**92-97.

8. Kubo A, Akdogan G, Nakaya M, Shojo A, Suzuki S, Satoh H, Kitamura S: **Structure, Physical, and Digestive Properties of Starch from wx ae Double-Mutant Rice.** *Journal of Agricultural and Food Chemistry* 2010, **58:**4463-4469.

9. Nishi A, Nakamura Y, Tanaka N, Satoh H: **Biochemical and Genetic Analysis of the Effects of Amylose-Extender Mutation in Rice Endosperm.** *Plant Physiology* 2001, **127:**459-472.

10. Cicero AF, Gaddi A: **Rice bran oil and gamma-oryzanol in the treatment of hyperlipoproteinaemias and other conditions.** *Phytotherapy Research* 2001, **15:**277-289.

11. Sakamoto K, Tabata T, Shirasaki K, Inagaki T, Nakayama S: **Effects of gamma-oryzanol and cycloartenol ferulic acid ester on cholesterol diet induced hyperlipidemia in rats.** *Japanese Journal of Pharmacology* 1987, **45:**559-565.

12. Grundy SM: **Metabolic syndrome: connecting and reconciling cardiovascular and diabetes worlds.** *Journal of the American College of Cardiology* 2006, **47:**1093-1100.

13. Abeywardena MY, Patten GS: **Role of ω3 long-chain polyunsaturated fatty acids in reducing cardio-metabolic risk factors.** *Endocr Metab Immune Disord Drug Targets* 2011, **11:**232-246.

14. Matsumoto K, Yokoyama S: **Induction of uncoupling protein-1 and -3 in brown adipose tissue by kaki-tannin in type 2 diabetic NSY/Hos mice.** *Food and Chemical Toxicology* 2012, **50:**184-190.

15. Robertson MD, Bickerton AS, Dennis AL, Vidal H, Frayn KN: **Insulin-sensitizing effects of dietary resistant starch and effects on skeletal muscle and adipose tissue metabolism.** *American Journal of Clinical Nutrition* 2005, **82:**559-567.

16. Behall KM, Scholfield DJ, Hallfrisch JG, Liljeberg-Elmståhl HG: **Consumption of both resistant starch and beta-glucan improves postprandial plasma glucose and insulin in women.** *Diabetes Care* 2006,

29:976-981.

17. Zhou J, Martin RJ, Tulley RT, Raggio AM, McCutcheon KL, Shen L, Danna SC, Tripathy S, Hegsted M, Keenan MJ: **Dietary resistant starch upregulates total GLP-1 and PYY in a sustained day-long manner through fermentation in rodents.** *American Journal of Physiology - Endocrinology and Metabolism* 2008, **295:**E1160-E1166.

18. Lattimer JM, Haub MD: **Effects of dietary fiber and its components on metabolic health.** *Nutrients* 2010, **2:**1266-1289.

19. Miller A, Frenzel T, Schmarr HG, Engel KH: **Coupled liquid chromatography-gas chromatography for the rapid analysis of gamma-oryzanol in rice lipids.** *Journal of Chromatography A* 2003, **985:**403-410.

20. Xu Z, Godber JS: **Purification and identification of components of γ-oryzanol in rice bran oil.** *Journal of Agricultural and Food Chemistry* 1999, **47:**2724-2728.

21. Wilson TA, Nicolosi RJ, Woolfrey B, Kritchevsky D: **Rice bran oil and oryzanol reduce plasma lipid and lipoprotein cholesterol concentrations and aortic cholesterol ester accumulation to a greater extent than ferulic acid in hypercholesterolemic hamsters.** *Journal of Nutritional Biochemistry* 2007, **18:**105-112.

22. Cheng HH, Ma CY, Chou TW, Chen YY, Lai MH: **Gamma-oryzanol ameliorates insulin resistance and hyperlipidemia in rats with streptozotocin/nicotinamide-induced type 2 diabetes.** *International Journal for Vitamin and Nutrition Research* 2010, **80:**45-53.

23. Islam MS, Murata T, Fujisawa M, Nagasaka R, Ushio H, Bari AM, Hori M, Ozaki H: **Anti-inflammatory effects of phytosteryl ferulates in colitis induced by dextran sulphate sodium in mice.** *British Journal of Pharmacology* 2008, **154:**812-824.

24. Shibata M, Yasuda B: **New experimental congenital diabetic mice (N.S.Y. mice).** *Tohoku Journal of Experimental Medicine* 1980, **130:**139-142.

25. Kobayashi M, Ikegami H, Fujisawa T, Nojima K, Kawabata Y, Noso S, Babaya N, Itoi-Babaya M, Yamaji K, Hiromine Y, et al: **Prevention and treatment of obesity, insulin resistance, and diabetes by bile acid-binding resin.** *Diabetes* 2007, **56:**239-247.

26. Itoi-Babaya M, Ikegami H, Fujisawa T, Ueda H, Nojima K, Babaya N, Kobayashi M, Noso S, Kawaguchi Y, Yamaji K, et al: **Fatty liver and obesity: Phenotypically correlated but genetically distinct traits in a mouse model of type 2 diabetes.** *Diabetologia* 2007, **50:**1641-1648.

27. Babaya N, Fujisawa T, Nojima K, Itoi-Babaya M, Yamaji K, Yamada K, Kobayashi M, Ueda H, Hiromine Y, Noso S, Ikegami H: **Direct evidence for susceptibility genes for type 2 diabetes on mouse chromosomes 11 and 14.** *Diabetologia* 2010, **53:**1362-1371.

28. Yu S, Nehus ZT, Badger TM, Fang N: **Quantification of vitamin E and gamma-oryzanol components in rice germ and bran.** *Journal of Agricultural and Food Chemistry* 2007, **55:**7308-7313.

29. Yoshie A, Kanda A, Nakamura T, Igusa H, Hara S: **Comparison of gamma-oryzanol contents in crude rice bran oils from different sources by various determination methods.** *Journal of Oleo Science* 2009, **58:**511-518.

30. Bligh EG, Dyer WJ: **A rapid method of total lipid extraction and purification.** *Canadian Journal of Biochemistry and Physiology* 1959, **37:**911-917.

31. Matsumoto K, Hiraiwa N, Yoshiki A, Ohnishi M, Kusakabe M: **PDGF receptor-alpha deficiency in glomerular mesangial cells of tenascin-C knockout mice.** *Biochemical and Biophysical Research Communications* 2002, **290:**1220-1227.

32. Wang TD, Chen WJ, Chien KL, Seh-Yi Su SS, Hsu HC, Chen MF, Liau CS, Lee YT: **Efficacy of cholesterol levels and**

ratios in predicting future coronary heart disease in a Chinese population. *American Journal of Cardiology* 2001, **88:**737-743.

33. Clifton JD, Lucumi E, Myers MC, Napper A, Hama K, Farber SA, Smith AB, Huryn DM, Diamond SL, Pack M: **Identification of novel inhibitors of dietary lipid absorption using zebrafish.** *PLoS One* 2010, **5:**e12386.

34. Calkin AC, Tontonoz P: **Transcriptional integration of metabolism by the nuclear sterol-activated receptors LXR and FXR.** *Nature Reviews Molecular Cell Biology* 2012, **13:**213-224.

35. Schwarz M, Wright AC, Davis DL, Nazer H, Björkhem I, Russell DW: **The bile acid synthetic gene 3beta-hydroxy-Delta(5)-C(27)-steroid oxidoreductase is mutated in progressive intrahepatic cholestasis.** *Journal of Clinical Investigation* 2000, **106:**1175-1184.

36. Hauptman J: **Orlistat: selective inhibition of caloric absorption can affect long-term body weight.** *Endocrine* 2000, **13:**201-206.

37. Tsujita T, Takaichi H, Takaku T, Sawai T, Yoshida N, Hiraki J: **Inhibition of lipase activities by basic polysaccharide.** *Journal of Lipid Research* 2007, **48:**358-365.

38. Tsutsumi K, Kawauchi Y, Kondo Y, Inoue Y, Koshitani O,

Kohri H: **Water extract of defatted rice bran suppresses visceral fat accumulation in rats.** *Journal of Agricultural and Food Chemistry* 2000, **48:**1653-1656.

39. Englyst KN, Liu S, Englyst HN: **Nutritional characterization and measurement of dietary carbohydrates.** *European Journal of Clinical Nutrition* 2007, **61 Suppl 1:**S19-39.

40. De Silva A, Bloom SR: **Gut Hormones and Appetite Control: A Focus on PYY and GLP-1 as Therapeutic Targets in Obesity.** *Gut and Liver* 2012, **6:**10-20.

41. Espenshade PJ: **SREBPs: sterol-regulated transcription factors.** *Journal of Cell Science* 2006, **119:**973-976.

42. Christakos S, Ajibade DV, Dhawan P, Fechner AJ, Mady LJ: **Vitamin D: metabolism.** *Endocrinology Metabolism Clinics of North America* 2010, **39:**243-253, table of contents.

43. Bu FX, Armas L, Lappe J, Zhou Y, Gao G, Wang HW, Recker R, Zhao LJ: **Comprehensive association analysis of nine candidate genes with serum 25-hydroxy vitamin D levels among healthy Caucasian subjects.** *Human Genetics* 2010, **128:**549-556.

44. Mathieu C, Gysemans C, Giulietti A, Bouillon R: **Vitamin D and diabetes.** *Diabetologia* 2005, **48:**1247-1257.

45. Satoh H, Omura T: **Induction of Mutation by the Treatment of Fertilized Egg Cell with N-methyl-N-nitrosourea in Rice.** *Journal of the Faculty of Agriculture, Kyushu University* 1979, **24:**165-174.

46. Matsumoto K, Maekawa M, Nakaya M, Takemitsu H, Satoh H, Kitamura S: **Wx/ae Double-Mutant Brown Rice Prevents the Rise in Plasma Lipid and Glucose Levels in Mice.** *Bioscience, Biotechnology, and Biochemistry* 2012, **76:**2112-2117.

47. Berry CS: **Resistant starch: Formation and measurement of starch that survives exhaustive digestion with amylolytic enzymes during the determination of dietary fibre.** *Journal of Cereal Science* 1986, **4:**301-314.

48. Englyst HN, Macfarlane GT: **Breakdown of resistant and readily digestible starch by human gut bacteria.** *Journal of the Science of Food and Agriculture* 1986, **37:**699-706.

49. Raben A, Tagliabue A, Christensen N, Madsen J, Holst J, Astrup A: **Resistant starch: the effect on postprandial glycemia, hormonal response, and satiety.** *American Journal of Clinical Nutrition* 1994, **60:**544-551.

50. Reader.D, Johnson.M.L, Hollander.P, Franz.M: **Response of resistant starch in a food bar vs. two commercially available bars in persons with type II diabetes mellitus.** *Diabetes* 1997, **46:**245A.

51. Han K, Fukushima M, Kato T, Kojima M, Ohba K, Shimada K, Sekikawa M, Nakano M: **Enzyme-resistant fractions of beans lowered serum cholesterol and increased sterol excretions and hepatic mRNA levels in rats.** *Lipids* 2003, **38:**919-924.

52. Martinez-Flores HE, Kil Chang Y, Martinez-Bustos F, Sgarbieri V: **Effect of high fiber products on blood lipids and lipoproteins in hamsters.** *Nutrition Research* 2004, **24:**85-93.

53. Higgins J, Higbee D, Donahoo W, Brown I, Bell M, Bessesen D: **Resistant starch consumption promotes lipid oxidation.** *Nutrition and Metabolism* 2004, **1:**8.

54. Nugent AP: **Health properties of resistant starch.** *Nutrition Bulletin* 2005, **30:**27-54.

55. Cicero AF, Gaddi A: **Rice bran oil and gamma-oryzanol in the treatment of hyperlipoproteinaemias and other conditions.** *Phytotherapy Research* 2001, **15:**277-289.

56. Sakamoto K, Tabata T, Shirasaki K, Inagaki T, Nakayama S: **Effects of gamma-oryzanol and cycloartenol ferulic acid ester on cholesterol diet induced hyperlipidemia in rats.** *Japanese Journal of Pharmacology* 1987, **45:**559-565.

57. Islam MS, Murata T, Fujisawa M, Nagasaka R, Ushio H, Bari AM, Hori M, Ozaki H: **Anti-inflammatory effects of**

phytosteryl ferulates in colitis induced by dextran sulphate sodium in mice. *British Journal of Pharmacology* 2008, **154:**812-824.

58. Kozuka C, Yabiku K, Sunagawa S, Ueda R, Taira S-i, Ohshiro H, Ikema T, Yamakawa K, Higa M, Tanaka H, et al: **Brown Rice and Its Component, γ-Oryzanol, Attenuate the Preference for High-Fat Diet by Decreasing Hypothalamic Endoplasmic Reticulum Stress in Mice.** *Diabetes* 2012.

59. Butsat S, Siriamornpun S: **Antioxidant capacities and phenolic compounds of the husk, bran and endosperm of Thai rice.** *Food Chemistry* 2010, **119:**606-613.

60. Zaima N, Goto-Inoue N, Hayasaka T, Setou M: **Application of imaging mass spectrometry for the analysis of Oryza sativa rice.** *Rapid Communications in Mass Spectrometry* 2010, **24:**2723-2729.

61. Matsushima Y, Hayashi S-i, Tachibana M: **Spontaneously hyperlipidemic (SHL) mice: Japanese wild mice with apolipoprotein E deficiency.** *Mammalian Genome* 1999, **10:**352-357.

62. Ide T, Takahashi Y, Kushiro M, Tachibana M, Matsushima Y: **Effect of n-3 fatty acids on serum lipid levels and hepatic fatty acid metabolism in BALB/c.KOR-Apoeshl mice deficient in apolipoprotein E expression.** *Journal of*

Nutritional Biochemistry 2004, **15:**169-178.

63. Mahley R: **Apolipoprotein E: cholesterol transport protein with expanding role in cell biology.** *Science* 1988, **240:**622-630.

64. Matsushima Y, Sakurai T, Ohoka A, Ohnuki T, Tada N, Asoh Y, Tachibana M: **Four strains of spontaneously hyperlipidemic (SHL) mice: phenotypic distinctions determined by genetic backgrounds.** *Journal of Atherosclerosis and Thrombosis* 2001, **8:**71-79.

65. Zhang SH, Reddick RL, Piedrahita JA, Maeda N: **Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E.** *Science* 1992, **258:**468-471.

66. Fujino Y, Miyazawa T: **Neutral Lipids Present in Starch of Uruchi and Mochi Rice.** *Starch - Stärke* 1976, **28:**414-420.

67. Zullaikah S, Melwita E, Ju YH: **Isolation of oryzanol from crude rice bran oil.** *Bioresource Technology* 2009, **100:**299-302.

68. Chen C-R, Wang L-Y, Wang C-H, Ho W-J, Chang C-MJ: **Supercritical carbon dioxide extraction of rice bran oil and column partition fractionation of γ-oryzanols.** *Separation and Purification Technology* 2008, **61:**358-365.

69. Yoshie A, Kanda A, Nakamura T, Igusa H, Hara S: **Comparison of gamma-oryzanol contents in crude rice bran oils from different sources by various determination methods.** *Journal of Oleo Science* 2009, **58:**511-518.

70. Reeves PG: **Components of the AIN-93 diets as improvements in the AIN-76A diet.** *Journal of Nutrition* 1997, **127:**838S-841S.

71. Maeda N, Shimomura I, Kishida K, Nishizawa H, Matsuda M, Nagaretani H, Furuyama N, Kondo H, Takahashi M, Arita Y, et al: **Diet-induced insulin resistance in mice lacking adiponectin/ACRP30.** *Nature Medicine* 2002, **8:**731-737.

72. Bligh EG, Dyer WJ: **A rapid method of total lipid extraction and purification.** *Canadian Journal of Biochemistry and Physiology* 1959, **37:**911-917.

73. Yogi M: **Studies on the difference of the cholesterol value as between the o PA method and the Zak Henly modification method (Japanese).** *IRYO - Japanese Journal of National Medical Services* 1974, **28:**367-371+347.

74. Fletcher MJ: **A colorimetric method for estimating serum triglycerides.** *Clinica Chimica Acta* 1968, **22:**393-397.

75. Han KH, Fukushima M, Shimizu K, Kojima M, Ohba K, Tanaka A, Shimada K, Sekikawa M, Nakano M: **Resistant starches of beans reduce the serum cholesterol concentration in rats.** *Journal of Nutritional Science and Vitaminology*2003, **49:**281-286.

76. Han KH, Iijuka M, Shimada K, Sekikawa M, Kuramochi K, Ohba K, Ruvini L, Chiji H, Fukushima M: **Adzuki resistant starch lowered serum cholesterol and hepatic 3-hydroxy-3-methylglutaryl-CoA mRNA levels and increased hepatic LDL-receptor and cholesterol 7alpha-hydroxylase mRNA levels in rats fed a cholesterol diet.** *British Journal of Nutrition* 2005, **94:**902-908.

77. Chen CW, Cheng HH: **A rice bran oil diet increases LDL-receptor and HMG-CoA reductase mRNA expressions and insulin sensitivity in rats with streptozotocin/nicotinamide-induced type 2 diabetes.** *Journal of Nutrition* 2006, **136:**1472-1476.

78. Espenshade PJ: **SREBPs: sterol-regulated transcription factors.** *Journal of Cell Science* 2006, **119:**973-976.

79. Wright RS, Anderson JW, Bridges SR: **Propionate inhibits hepatocyte lipid synthesis.** *Proceedings of The Society for Experimental Biology and Medicine* 1990, **195:**26-29.

80. Hara H, Haga S, Aoyama Y, Kiriyama S: **Short-chain fatty acids suppress cholesterol synthesis in rat liver and**

intestine. *Journal of Nutrition* 1999, **129:**942-948.

81. Barter P: **The role of HDL-cholesterol in preventing atherosclerotic disease.** *European Heart Journal, Supplements* 2005, **7:**F4-F8.

82. Homan R, Grossman JE, Pownall HJ: **Differential effects of eicosapentaenoic acid and oleic acid on lipid synthesis and secretion by HepG2 cells.** *Journal of Lipid Research* 1991, **32:**231-241.

83. Imae R, Inoue T, Kimura M, Kanamori T, Tomioka NH, Kage-Nakadai E, Mitani S, Arai H: **Intracellular phospholipase A1 and acyltransferase, which are involved in Caenorhabditis elegans stem cell divisions, determine the sn-1 fatty acyl chain of phosphatidylinositol.** *Molecular Biology of the Cell* 2010, **21:**3114-3124.

84. Ichimura A, Hirasawa A, Poulain-Godefroy O, Bonnefond A, Hara T, Yengo L, Kimura I, Leloire A, Liu N, Iida K, et al: **Dysfunction of lipid sensor GPR120 leads to obesity in both mouse and human.** *Nature* 2012, **483:**350-354.

85. Nunez EA: **Biological complexity is under the 'strange attraction' of non-esterified fatty acids.** *Prostaglandins, Leukotrienes and Essential Fatty Acids* 1997, **57:**107-110.

86. Haber EP, Ximenes HM, Procópio J, Carvalho CR, Curi R,

Carpinelli AR: **Pleiotropic effects of fatty acids on pancreatic beta-cells.** *Journal of Cellular Physiology* 2003, **194:**1-12.

87. Cao H, Gerhold K, Mayers JR, Wiest MM, Watkins SM, Hotamisligil GS: **Identification of a lipokine, a lipid hormone linking adipose tissue to systemic metabolism.** *Cell* 2008, **134:**933-944.

88. Ghule BV, Ghante MH, Saoji AN, Yeole PG: **Antihyperlipidemic effect of the methanolic extract from Lagenaria siceraria Stand. fruit in hyperlipidemic rats.** *Journal of Ethnopharmacology* 2009, **124:**333-337.

89. Spayd RW, Bruschi B, Burdick BA, Dappen GM, Eikenberry JN, Esders TW, Figueras J, Goodhue CT, LaRossa DD, Nelson RW, et al: **Multilayer film elements for clinical analysis: applications to representative chemical determinations.** *Clinical Chemistry* 1978, **24:**1343-1350.

90. Nakaya M, Shojo A, Hirai H, Matsumoto K, Kitamura S: **Hypolipidemic effects of starch and γ-oryzanol from wx/ae double-mutant rice on BALB/c.KOR-Apoe(shl) mice.** *Bioscience, Biotechnology, and Biochemistry* 2013, **77:**1435-1440.

91. Moreau RA: **Lipid analysis via HPLC with a charged aerosol detector.** *Lipid Technology* 2009, **21:**191-194.

92. Dixon RW, Peterson DS: **Development and testing of a detection method for liquid chromatography based on aerosol charging.** *Analytical Chemistry* 2002, **74:**2930-2937.

93. Moreau RA: **The analysis of lipids via HPLC with a charged aerosol detector.** *Lipids* 2006, **41:**727-734.

94. Porter NA: **A perspective on free radical autoxidation: the physical organic chemistry of polyunsaturated fatty acid and sterol peroxidation.** *Journal of Organic Chemistry* 2013, **78:**3511-3524.

95. Yin H, Xu L, Porter NA: **Free radical lipid peroxidation: mechanisms and analysis.** *Chemical Reviews* 2011, **111:**5944-5972.

96. Salvayre R, Auge N, Benoist H, Negre-Salvayre A: **Oxidized low-density lipoprotein-induced apoptosis.** *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids* 2002, **1585:**213-221.

97. Marnett LJ: **Lipid peroxidation-DNA damage by malondialdehyde.** *Mutation Research* 1999, **424:**83-95.

98. Furukawa S, Hirano T: **Rapid stimulation of apolipoprotein B secretion by oleate is not associated with cholesteryl ester biosynthesis in HepG2 cells.** *Biochimica et Biophysica Acta - Lipids and Lipid*

Metabolism 1993, **1170:**32-37.